The Study of the Antigenic Determinants of Trichosanthin

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Abstract

Trichosanthin (TCS) is a ribosome-inactivating protein (RIP) which is an active component of Tian hua fen (THF), the root tuber of the Chincse medicinal herb *Trichosanthes kirilowii* Maximowicz. TCS has been used in abortifacient purposes and the clinical trials on HIV-infected patients. However, hypersensitivity response is elicited in certain cases. My project concerns with location of the antigenic determinants in this protein.

Transposon mediated deletion mutagenesis and protein fragmentation have been used to find the antigenic determinants of TCS. It was found that the polyclonal antibody, classical monoclonal antibody and Fab fragment employed recognized a discontinuous epitope on TCS. The forty two clones of Fab fragments produced by recombinant method were found to produce similar binding pattern towards the deletion mutants. The binding pattern is also identical to that of the monoclonal antibody used. The antigenic sites of this protein were shown to locate between amino acids residues 1-21, 21-73 and 154-247. Residues 102-153 of TCS was found to recognize only by the anti-TCS polyclonal antibody. Residues 74-100 did not show antigenicity with the antibodies used.

Contents

Abbreviations

ATP	adenosine 5' -triphosphate
bp	base pair (s)
BSA	bovine serum albumin
cDNA	complementary DNA
dATP	deoxyadenosine 5' triphosphate
dCTP	deoxycytosine 5' triphosphate
dGTP	deoxyguanosine 5' triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide 5' triphosphate
EDTA	ethylenediaminetetraacetic acid
HIV	human immunodeficiency virus
IPTG	isoproply β-D-thiogalactopyranoside
kb	kilobase (s)
PAGE	polyacrylamide gel electrophoresis
RIP	ribosome-inactivating protein
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
TCS	Trichosanthin
TEMED	N,N,N,N-tetramethyl ethylene diamine
Tris	Tris [hydroxymethyl] aminomethane
TTP	thymidine 5' triphosphate
UV	ultraviolet

Chapter One

General Introduction

Trichosanthin (TCS) is a ribosome-inactivating protein (RIP) which is the active component of Tian hua fen (THF), the root tuber of the Chinese medicinal herb; *Trichosanthes kirilowii* Maximowicz of Cucurbitaceae family. THF had been used for abortifacient purpose in China for centuries. In the 70s, the active ingredient of THF, TCS, was found and purified (Wang *et al.*, 1976). This has led to the initiation of the investigation for the use of TCS as abortifiacient agents for birth control in China (Liu *et al.*, 1985; Jin, 1985).

TCS has also been used in the treatment of trophoblastic tumours, ectopic pregnancy and retention of dead fetuses (Jin, 1985; Egarter, *et al.*, 1991; Huang, 1987). These properties are due to the specific cytotoxicity to trophoblasts (Anonymous, 1976). In recent years, TCS has also been found to inhibit the replication of type 1 human immuno-deficiency virus (HIV-1) *in vitro* with the additional property of destroying HIV-infected macrophages (McGrath *et al.*, 1989). This has led to the phases I & II clinical trials to test for the treatment of AIDS by the TCS protein (Byers *et al.*, 1990; Pinching, 1990). However, one problem in using TCS in clinical treatment is that TCS can elicit hypersensitivity response (Byers *et al.*, 1990; Pinching, 1990; Jin, 1985; Guowu *et al.*, 1985) and limited information on the antigenic sites of TCS protein has been found. Therefore this study involves the elucidation of the antigenic determinants of TCS.

1.1 Chemical and Biological Properties of TCS

1.1.1 Chemical properties

TCS purified from root tubers of *T. kirilowii* Maxim was free of carbohydrate and phosphate groups. It is a highly basic protein which has a pI value of 9.4 as determined by glycerol gradient isoelectric focusing method (Jin, 1985; Wang, 1985).

In recent years the cDNA (Shaw *et al.*, 1991) and the genomic DNA (Chow *et al.*, 1990) of TCS have been cloned. The cDNA of TCS encodes for a prepropeptide of 289 amino acids (Shaw *et al.*, 1991). The first 23 residues resemble a secretary signal peptide and the last 19 amino acid residues comprise a propeptide which is cleaved in post-translational processing (Chow *et al.*, 1991). It has been shown that the propeptide keeps the cytotoxic protein in less active form (Zhu *et al.*, 1992). Mature TCS consists of 247 amino acids (Fig. 1.1) (Chow *et al.*, 1990; Shaw *et al.*, 1991) which contains no cysteine residues, therefore no disulfide linkage can be formed. The molecular weight of TCS as calculated from the amino acid sequence is 27140 (Chow *et al.*, 1990; Shaw *et al.*, 1991). An efficient expression system has been developed for the expression of TCS in *E. coli* (Zhu *et al.*, 1992).

Recently, the three-dimensional structure of TCS has been solved by X-ray crystallography to 1.7Å resolution (Dong *et al.*, 1994) and Fig 1.2 shows the 3-D structure of TCS protein. The 3-D structures of ricin A chain and TCS are very similar (Fig. 1.3).

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  1  Met Asp Val Ser Phe Arg Leu Ser Gly Ala Thr Ser Ser Ser Tyr Gly Val Phe Ile Ser     20
  1  ATG GAT GTT AGC TTC CGT TTA TCA GGT GCA ACA AGC AGT TCC TAT GGA GTT TTC ATT TCA     60

 21  Asn Leu Arg Lys Ala Leu Pro Asn Glu Arg Lys Leu Tyr Asp Ile Pro Leu Leu Arg Ser     40
 61  AAT CTG AGA AAA GCT CTT CCA AAT GAA AGG AAA CTG TAC GAT ATC CCT CTG TTA CGT TCC     120

 41  Ser Leu Pro Gly Ser Gln Arg Tyr Ala Leu Ile His Leu Thr Asn Tyr Ala Asp Glu Thr     60
121  TCT CTT CCA GGT TCT CAA CGC TAC GCA TTG ATC CAT CTC ACA AAT TAC GCC GAT GAA ACC     180

 61  Ile Ser Val Ala Ile Asp Val Thr Asn Val Tyr Ile Met Gly Tyr Arg Ala Gly Asp Thr     80
181  ATT TCA GTG GCC ATA GAC GTA ACG AAC GTC TAT ATT ATG GGA TAT CGC GCT GGC GAT ACT     240

 81  Thr Tyr Phe Phe Asn Glu Ala Ser Ala Thr Glu Ala Ala Lys Tyr Val Phe Lys Asp Ala     100
241  ACC TAT TTT TTC AAC GAG GCT TCT GCA ACA GAA GCT GCA AAA TAT GTA TTC AAA GAC GCT     300

101  Met Arg Lys Val Thr Leu Pro Tyr Ser Gly Asn Tyr Glu Arg Leu Gln Thr Ala Ala Gly     120
301  ATG CGA AAA GTT ACG CTT CCA TAT TCT GGC AAT TAC GAA AGG CTT CAA ACT GCT GCA GGC     360

121  Lys Ile Arg Glu Asn Ile Pro Leu Gly Leu Pro Ala Leu Asp Ser Ala Ile Thr Thr Leu     140
361  AAA ATA AGG GAA AAT ATT CCG CTT GGA CTC CCT GCT TTG GAC AGT GCC ATT ACC ACT TTG     420

141  Phe Tyr Tyr Asn Ala Asn Ser Ala Ala Ser Ala Leu Met Val Leu Ile Gln Ser Thr Ser     160
421  TTT TAC TAC AAC GCC AAT TCT GCT GCG TCG GCA CTT ATG GTA CTC ATT CAG TCG ACG TCT     480

161  Glu Ala Ala Arg Tyr Lys Phe Ile Glu Gln Gln Ile Gly Lys Arg Val Asp Lys Thr Phe     180
481  GAG GCT GCG AGG TAT AAA TTT ATT GAG CAA CAA ATT GGG AAG CGT GTT GAC AAA ACC TTC     540

181  Leu Pro Ser Leu Ala Ile Ile Ser Leu Glu Asn Ser Trp Ser Ala Leu Ser Lys Gln Ile     200
541  CTA CCA AGT TTA GCA ATT ATA AGT TTG GAA AAT AGT TGG TCT GCT CTC TCC AAG CAA ATT     600

201  Gln Ile Ala Ser Thr Asn Asn Gly Gln Phe Glu Ser Pro Val Val Leu Ile Asn Ala Gln     220
601  CAG ATA GCG AGT ACT AAT AAT GGA CAG TTT GAA AGT CCT GTT GTG CTT ATA AAT GCT CAA     660

221  Asn Gln Arg Val Thr Ile Thr Asn Val Asp Ala Gly Val Val Thr Ser Asn Ile Ala Leu     240
661  AAC CAA CGA GTC ACG ATA ACC AAT GTT GAT GCT GGA GTT GTA ACC TCC AAC ATC GCG TTG     720

241  Leu Leu Asn Arg Asn Asn Met Ala ***                                               249
721  CTG CTG AAT AGA AAC AAT ATG GCA TAG                                               747
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Fig.1.1 The DNA sequence and amino acid sequence of TCS.

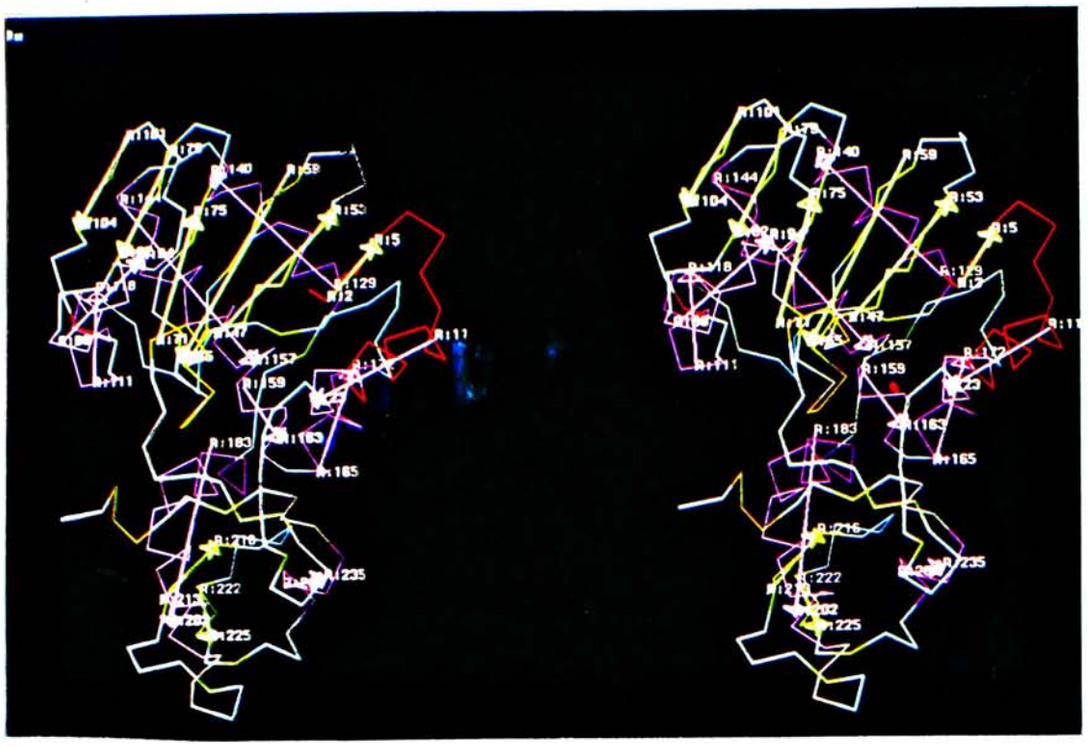


Fig. 1.2 The stereoimage of TCS protein determined by X-ray crystallography.

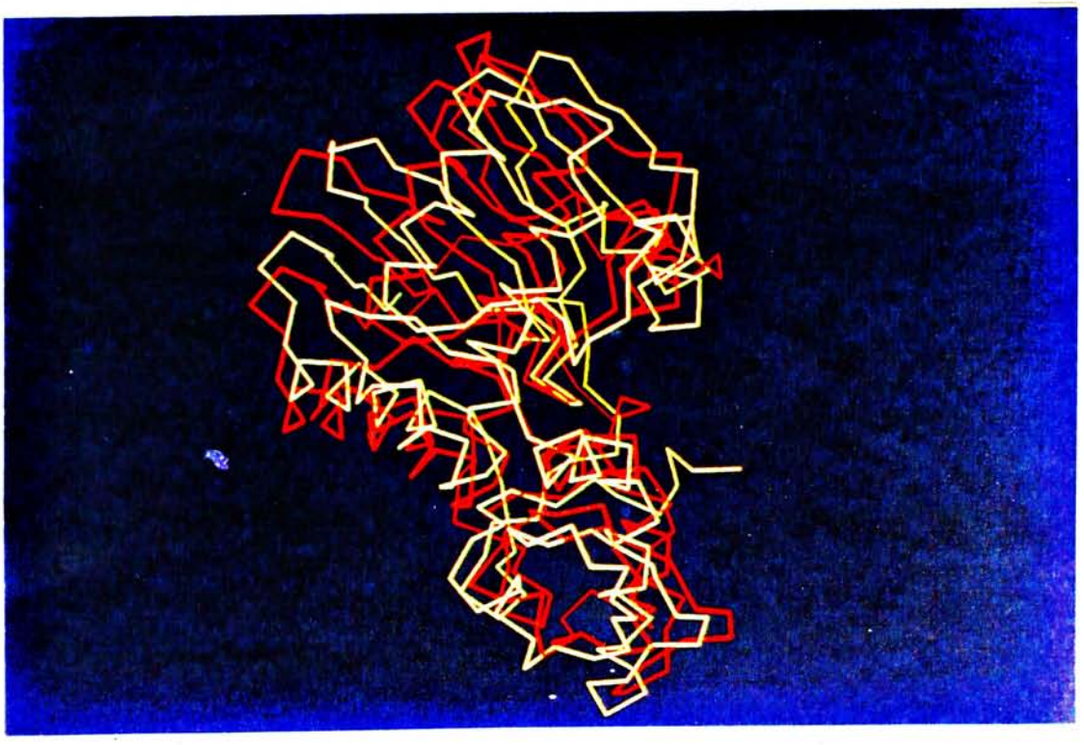


Fig. 1.3. Superimposition of TCS and ricin. Yellow and red lines represent α-backbone of TCS and ricin A-chain respectively.

1.1.2 Biological properties

TCS is a type I ribosome-inactivating protein (RIP). Type I RIPs are single chain basic proteins of molecular weight of 26-30kDa. They are potent inhibitors of protein synthesis in cell free system. The ribosome-inactivating activity of TCS is due to the N-glycosidase activity which specifically cleaves the N-glycosidic bond of A-4324 of 28s rRNA and releases the adenine residue (Endo and Tsungi, 1988; Zhang and Liu, 1992). It has been shown that the removal of A-4324 by ricin interferes with EF-1 (elongation factor 1) and GTP-dependent aminoacyl-tRNA binding to ribosome as well as the EF-2-catalysed GTP hydrolysis and translocation and hence disrupting the process of protein synthesis (Furutani *et al.*, 1992).

TCS elicits immune response such as the production of IgE and IgG (Ng *et al.*, 1992; Xu *et al.*, 1992) and hence limits its usefulness in clinical treatment. Some of its adverse reactions have been found in some practices in the induction of abortion and the treatment of AIDS after multiple administration of TCS to the patient. They include local inflammatory reaction, flu-like syndromes such as fever and myelalgias, and in extreme cases, fatal anaphylactic reaction (Lu and Jin, 1989 ; Kahn *et al.*, 1990; Ferrari *et al.*, 1991). The anaphylactic reaction may be due to the activation of complement by TCS via the alternative pathway (Chen and Ma, 1993).

1.2 Structure of Antigens

Since this study concerns with the investigation of the antigenic determinants of TCS, some basic information on the antigenic determinants will be introduced.

The antigenic specificities of a protein reside in restricted areas of the molecule known as antigenic determinants or epitopes, and are recognized by the combining sites or paratopes of certain immunoglobulin molecules (Van Regenmortel, 1991). Epitopes were originally classified as either sequential or conformational, however recently all epitopes are considered to be conformational but are classified as being either continuous or discontinuous. Continuous epitopes are defined as any short linear peptide fragments of the antigen that are able to bind to antibodies raised against the intact protein. Discontinuous epitopes are epitopes made up of residues in the protein that are not continuous in sequence but are brought together by the folding of the polypeptide chain (Van Regenmortel, 1991). Fig. 1.4 explains the difference between continuous and discontinuous epitopes recognized by the immunoglobulin. Epitopes may also be classified as structural or functional (Van Regenmortel, 1991). Structural epitopes refer to the amino acid residues that are involved in the contact of immunoglobulin and these epitopes are usually found out by X-ray crystallography of the antigen-antibody complex. Functional epitopes refer to the amino acid residues that are critical in the binding between the antigen and the antibody. These epitopes are usually found out by cross-reactivity binding between the antigenic peptide and the antibody or by site directed mutagenesis of amino acid residues in the protein. Usually structural epitopes contain more amino acid residues than the functional epitopes (Van Regenmortel, 1991).

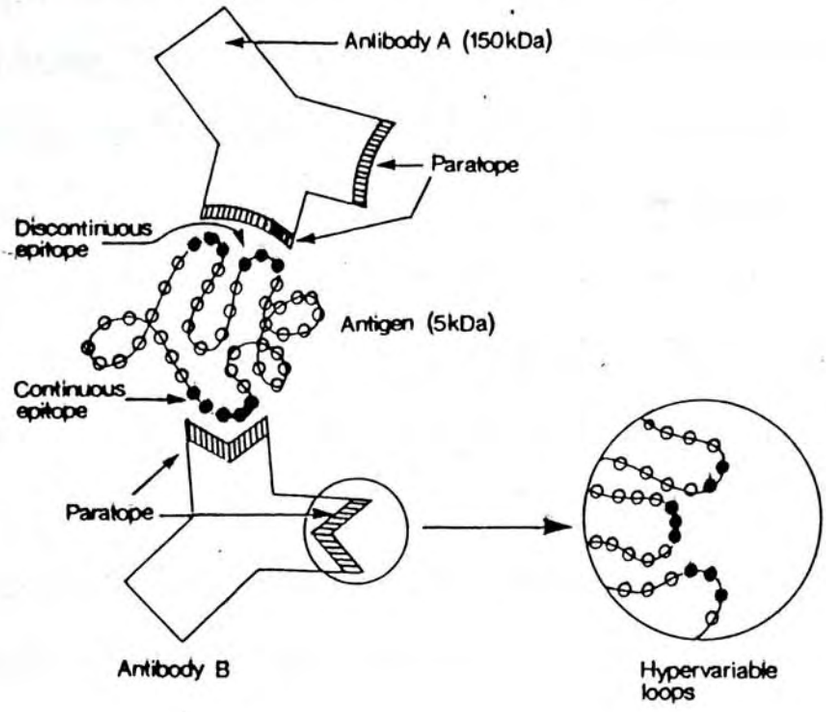


Fig. 1.4. Schematic representation of two antibodies interacting with a continuous and a discontinuous epitope of a protein antigen. Contact residues are indicated as solid circles (adopted from Van Regenmortel, 1991).

Many investigations have been done in order to find the essence of epitope on protein. However, up to now only some general characteristics of the epitope are known. Epitopes are generally on the surface of protein, more hydrophilic, more mobile and easily accessible (Westhof *et al.*, 1984; Hopp, 1986; Huang, 1990; Van Regenmortel, 1991). In recent years X-ray diffraction studies of complexes of monoclonal antibody Fab fragments with their protein antigens have been done. These results may give a clearer picture about the antigenic sites on the protein. One example is the antigenic sites on the lysozyme. Three epitopes have been analysed on its surface (Amit *et al.*, 1986; Padlan *et al.*, 1989; Sheriff *et al.*, 1987). In all cases, there is a large area of the protein surface (700 to 800Å^2) between 15 to 22 amino acid residues. All epitopes identified by X-ray crystallography so far are clearly discontinuous. The epitopes recognized by the anti-lysozyme D1.3 and HyHel-5 antibodies consist of two stretches of the lysozyme polypeptide chains (residues 18-27 and 116-129 for D1.3, and 41-53 and 67-70 for HyHel-5), whereas the lysozyme HyHEL-10 epitope consists of the exposed surface of a helix (residues 88-99) together with residues from several lysozme segments (residues 15-16, 20-21, 63, and 74-75). All of these epitopes are on the surface of the protein, more mobile and easily accessible.

1.3 Methods Used for the Mapping of Antigenic Sites on the Protein.

Most of our knowledge concerning the location of epitopes in proteins is by studying the antigenic cross-reactivity between the immunoglobulin and peptide fragments of the antigen. The following section summarizes some common approaches for mapping the antigenic sites on the protein.

X-ray crystallography

The first method of epitope mapping is the X-ray crystallography of the antigen-antibody complex. This method can find the structural epitopes of a protein and gives the clearest information on the epitopes of the protein. However it is not easy to produce the crystal of the antigen-antibody complex. Therefore only a small number of epitopes have been defined by this approach.

Cross-reactivity between synthetic peptide and immunoglobulin

Another method of epitope mapping makes use of the techniques of synthetic peptide. For example, Geysen *et al.* (1984) reported the use of synthetic peptide to probe for epitopes of a viral antigen. Hundreds of peptides (6 amino acids in length) which correspond to different region of the protein were synthesised and supported in a solid phase. These peptides were then analysed by enzyme-linked immunosorbent assay (ELISA) to look for the present of antigenic site on them. From this approach they have identified a seven amino acid peptide to be the antigenic site of the protein.

In another approach, antigenic site is predicted from cDNA sequence or amino acid sequence by antigenicity prediction algorithms. Then the antigenic fragment predicted by the algorithm is synthesised. The peptide is then injected to the animal to elicit immune response and to produce anti-peptide antibodies. If these antibodies can cross-react with the normal protein, the concerned peptide sequence is one of the antigenic sites of the protein (Mahale *et al.*, 1993).

In some cases the synthetic peptides are not injected into the animal. They are allowed to react with the antigen sensitive T cells, B cells or macrophages. Proliferation assays are then performed to see if the peptides can elicit cell proliferation activity. If the cells can proliferate in the presence of the peptide, this means that the peptide sequence is the antigenic site of the antigen recognized by the immunological cells (Wierz *et al.*, 1992; Kurate *et al.*, 1990) .

The advantage of using synthetic peptide is that usually continuous epitopes can be easily probed by this approach. However the major problem of this approach is that usually the degree of antigenic cross-reactivity observed with small peptide is very low.

Chemical modification

Burnens *et al.* (1987) used the characteristic that antibody bound to protein antigens decreases the rate of chemical modification of amino acid residues located at the epitope. Therefore by comparing the degree of acetylation of 18 lysine and threonine residues in free and antibody bound horse cytochrome, they identified the epitopes on the protein. This method can be used to map for discontinuous epitope, however it is not easy to find the suitable residues for chemical modification.

Epitope library

In this method, the cDNA of the interested protein is fragmented randomly by endonuclease. The fragment is then subcloned into expression vector to produce an epitope library. The library is then screened by the corresponding antibody to find out the positive clone that contains the antigenic site. The antigenic site is then located by DNA sequencing of the positive clone (Banting *et al.*, 1989). Nowadays, there is actually a kit provided from Novagen for this purpose. However intensive *in vitro* manipulation is required in this method.

Site specific mutagenesis

Site specific mutagenesis is used to mutate specific site of the cDNA. The antibody binding response toward wild-type protein and the mutants is compared. If a mutation results in the change of antibody binding, it is assumed that the mutated residue is involved in the structure of an epitope and hence the corresponding antigenic site can be located (Alexander *et al.*, 1992). This method can be used to define the discontinuous and functional epitopes up to several amino acids, however it is not easy to find the suitable region for mutagenesis.

Deletion mutagenesis

Deletion mutagenesis is done on cDNA of the interested protein by transposon mediated mutagenesis (Sedgwick *et al.*, 1991) or by exonuclease III (Gross and Rohemann, 1990). The mutants are then analysed by corresponding antibody. Antigenic site of the protein will be progressively lost as the expressed sequences are shortened. The corresponding antigenic site can be located by DNA sequencing of the

deletion mutants. This approach involves less *in vitro* manipulation but only the continuous epitope can be mapped by this method.

Analysis of chemical or enzymatic cleavage product of the protein

In this method the interested protein is cleaved by chemical or enzymatic method. The product is then analysed for the presence of antigenic site with the corresponding antibody. The location of the fragment can be found by analysing the protein sequence if the cleavage is specific in certain amino acid residues or by N-terminal determination of the corresponding antigenic fragment (Morris, 1989; Man *et al.*, 1991). This approach usually identifies a large antigenic fragment and further studies are required.

One of the characteristics of the epitope mapping is that all of the methods required the knowledge of cDNA sequence or amino acid sequence of the interested protein. Moreover the methods described have merits and drawbacks. Usually more than one of the approaches are required in the study of the antigenic determinants of a protein.

1.4 Objective and Strategy of the Study of the Antigenic Determinants of TCS

The present study is to find out the antigenic determinants of TCS. The TCS protein has been shown to be useful in a number of clinical treatment (for example abortion, anti-tumor and anti-HIV), however problems of hypersensitivity due to the immunological response toward TCS has been found. Up till now, the antigenic determinants of TCS have only been underly defined (Ke *et al.*, 1988). If the antigenic determinants of TCS are known, in the future the protein can be engineered to minimize its immunological side effects.

In this study the epitope mapping by transposon mediated deletion mutagenesis is followed (Sedgwick et al., 1991). This method has the advantages of little *in vitro* manipulation and able to locate the epitopes precisely. In transposon mediated deletion mutagenesis of the cDNA of TCS, transposon Tn*1000* is inserted randomly into an expression vector which contains the TCS cDNA sequence. Translational stop codons at both ends of Tn*1000* results in the production of shortened TCS proteins. Mutants which have lost the epitopes can be identified by Western blotting with antibodies. The precise location of the antigenic site can be determined by DNA sequencing with the use of Tn1000 specific primers.

Another approach of this study includes the use of proteolytic enzymes and cyanogen bromide to generate protein fragments from TCS protein. The fragments are then analysed by Western blotting to see which can interact with the anti-TCS antibody. The protein fragments which react with anti-TCS antibody are N-terminal sequenced and their size

determined by mass spectrometry to find out their locations on the TCS sequence.

Chapter Two

Materials and Methods

2.1 Bacterial Strains Used

The following table summarizes the bacterial strains of *Escherichia coli* used:

Bacterial strain	Genotype	Purpose in this study	Source
MH1345	*F^+ deoC srl::Tn10 recA1*	Donor cell in bacterial mating	S.G. Sedgwick
MH755	*F srl::Tn10 recA1 thr-1 leu-6 thi-1 lacY1 galK2 ara14 xyl14 mtl-1 proA2 his-4 argE3 rpl31 tsx33*	Recipient cell in bacterial mating	S.G. Sedgwick
DH5α	*F'/endA1 hsdR17($r_K^-m_K^+$) supE44 thi-1 recA1 gyrA* (Nal^r) *relA1 ▲(lacZYA-argF)U169 deoR (ϕ80dlac▲(lacZ)M15)*	For subcloning of mutant	Our laboratory
BL21 (DE3 pLysS)	*F ompT hsdS$_B$($r_K^-m_K^-$;an E.coli B strain)*	T7 RNA polymerase driven expression system	F.W. Studier

2.2 General Techniques

2.2.1 Extraction of DNA by phenol/chloroform

An equal volume of phenol/chloroform (1:1) was added to the mixture and mixed by gentle agitation. The mixture was then centrifuged at 12,000g (13,000rpm) for 5 minutes to separate the aqueous and organic layer. The aqueous layer was then transferred to a new microcentrifuge tube. The DNA was precipitated by ethanol.

2.2.2 Ethanol precipitation of DNA

0.1 volume of sodium acetate (3M pH5.2) and 3 volumes of ice-cooled 100% ethanol were added to the DNA solution. The DNA was precipitated at -70 °C for 30 min and was then pelleted at 12,000g (13,000rpm) for 15 minutes at 4 °C. The pellet was then washed with 70% ice-cooled ethanol once and dried in a SpeedVac (Savavt Instruments Inc.) machine. The DNA pellet was then dissolved in suitable amount of water.

2.2.3 Minipreparation of plasmid DNA

This method was adopted from Sambrook *et al.* (1989) with minor modification. A single bacterial colony was inoculated into 5ml of LB medium (section 2.16.1) containing the appropriate antibiotic in sterilized universal bottle. The culture was incubated overnight at 37°C with shaking at 250rpm. Bacterial pellet of 3ml culture was collected by centrifugation at 12,000g (13,000rpm) for 5 min in a microfuge. The supernatant was discarded, the pellet was re-centrifuged for one min and the last drop of

bacterial broth was taken out with a fine pipette. 0.3ml of P1 buffer (section 2.16.2) was used to resuspend the cells completely. Then 0.3ml of P2 buffer (section 2.16.2) was added and mixed immediately. The solution was allowed to stand at room temperature for 5 min. 0.3ml of P3 buffer (section 2.16.2) was added and mixed immediately with gentle agitation. The cell debris and chromosomal DNA were removed by centrifugation at 12,000g (13,000rpm) for 15 min in a microfuge. 0.9ml of supernatant was taken out carefully and added with 0.45ml isopropanol. The solution was kept on ice for 15 min and centrifuged for 15 minutes at 12,000g(13,000rpm). The pellet was washed with 0.5ml of 70% ethanol and dried by SpeedVac. The plasmid was dissolved in 30μl of water and stored at -20°C for later assay.

2.2.4 Preparation of plasmid DNA using Qiagen pack 100 Cartridge

This method was modified from the Qiagen Application Protocol (4th Edition). A single colony was inoculated into 400ml of LB medium containing the appropriate antibiotic. The culture was incubated overnight at 37°C with shaking at 250rpm. The bacteria were collected by centrifugation at 4,000g (5,000rpm) for 5 min at 4°C (Beckman J2-21 Centrifuge with JA-14 rotor). The pellet was resuspended in 10ml P1 buffer. After 10ml of P2 was added, the mixture was allowed to stand at room temperature for 5 min. Then 10ml of P3 buffer was then added to the mixture and mixed immediately but gently. The mixture was centrifuged at 4°C for 30 min (15,000g rotor JA 20). The supernatant was removed promptly and recentrifuged at 4°C for 10min (15,000g) to obtain a particle-free clear lysate. A supernatant of about 30ml was collected. A Qiagen-pack 100 column (Qiagen Inc.) was equilibrated

with 3ml of QBT (750mM NaCl, 50mM MOPS, 15% ethanol, pH7.0, 0.15% Triton X-100). The flow was maintained by gravity. 1/3 volume (about 10ml) of the supernatant collected by centrifugation was applied to the column. The column was then washed with 10ml QC (1.0M NaCl, 50mM MOPS, 15% ethanol, pH7.0). The plasmid DNA adsorbed onto the column was eluted by passing through 5ml of QF (1.25M NaCl, 50mM MOPS, 15% ethanol, pH8.2). The above procedure was repeated twice to purify the plasmid DNA in the remaining supernatant.

The plasmid DNA eluted from the above steps was recovered by precipitation with 0.7 volumes of isopropanol. The mixture was allowed to incubate on ice for 15 min and then the DNA was recovered by centrifugation at 12,000g (13,00rpm) for 20 min at 4°C. The pellet was washed with 70% ethanol and dried by SpeedVac. The DNA was dissolved in 200µl of water and stored at -20°C.

2.2.5 Preparation of plasmid DNA using Magic™ Minipreps DNA Purification kit from Promega

The following procedure is based on the Technical Bulletin provided by the Purification kit. A single colony was inoculated into 5ml of LB medium containing the appropriate antibiotic in a universal bottle. The culture was incubated overnight at 37°C with shaking at 250rpm. Bacterial pellet was collected by centrifugation at 12,000g (13,000rpm) for 5 min in a microfuge. The bacterial pellet was resuspended in 200µl of P1. Then 200µl of P2 was

added and the tube was inverted several times to mix the solution. 200µl of P3 was added and the mixture was mixed by gentle agitation. Cell debris and chromosomal DNA were removed by centrifugation at 12,000g (13,000rpm) for 15 min in a microfuge. The supernatant was transferred to a new microcentrifuge tube. 1ml of the Magic™ Minipreps DNA Purification resin was added to the supernatant and the mixture was mixed by inverting the tube. A Magic™ Minicolumn was prepared by connecting the column to a 3ml disposable syringe with its plunger removed. The resin/DNA mixture was pipetted into the syringe barrel. The syringe plunger was inserted slowly and gently to push the slurry into the Minicolumn. Then the column was washed with 2ml of Column Wash Solution. Then the Minicolumn was transferred to a new 1.5ml microcentrifuge tube and centrifuged for 20 sec at 12,000g (13,000rpm) in a microfuge to dry the resin. The Minicolumn was then transferred to a new microcentrifuge tube and 50µl of water was applied to the Minicolumn. After incubation for 1 min, the Minicolumn was centrifuged for 20 sec at 12,000g (13,000rpm) to elute the DNA. The plasmid was stored at -20°C for later used.

2.2.6 Preparation and transformation of *Escherichia coli* competent Cells

The following method was adopted from Sambrook *et al.* (1989) with modification. *Escherichia coli* cells of the concerned strain was streaked directly from a frozen stock onto the surface of an ϕb plate (section 2.16.1). The plate was incubated overnight at 37°C and single colony from the plate was transferred to 5ml ϕb medium (section 2.16.1). The culture was

incubated at 37°C with shaking until $O.D._{600}$ reached about 0.3. The 5ml culture was then poured into 100ml ϕb medium and were continued to shake at 37°C for 2-3 hrs until $O.D._{600}$ was about 0.45. The culture was then incubated on ice for 5 min. The bacterial cells were collected by centrifugation at 4,000g (5,000rpm) for 10 min at 4 °C (Beckman J2-21 Centrifuge with JA-14 rotor). After standing the tubes in an inverted position for 1 min to remove the last traces of medium, the pellet was resuspended in 40 ml of TfbI (30mM KAc, 100mM $RbCl_2$, 10mM $CaCl_2$, 50mM $MnCl_2$ and 15% glycerol). The cells were incubated on ice for 5 min. Then the cells were collected by centrifugation at 4,000g for 10 min at 4 °C (Beckman J2-21 Centrifuge with JA 20 rotor). The supernatant was discarded and the cell pellet was resuspended in 4ml of TfbII (10mM MOPS, 75mM $CaCl_2$, 10mM $RbCl_2$ and 15% glycerol, adjust pH to 6.5 with KOH). The cells were kept on ice for 15 min and the competent cell suspension was dispensed into aliquots of 200µl each. The aliquots were frozened in liquid nitrogen and stored at -70 °C until use.

For the transformation of the competent cells, the cells were put on ice until just thawed and about 100ng of DNA was added, and mixed gently. The tube was left on ice for 30 min and then heat shocked at 42 °C for exactly 90 sec. The tube was then immediately returned to ice for 2 min. Then 4 volumes of LB medium was added into the tube and incubated at 37 °C for 50 min with constant shaking to allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid. After the incubation, 50-100µl of the transformed culture was spreaded onto a LB agar plate with an

appropriate antibiotic. When the liquid had been absorbed, the plates were inverted and incubated at 37 °C overnight.

2.2.7 Agarose gel electrophoresis of DNA

The agarose gel was prepared with 0.8-1.2% (w/v) agarose (BioRad) dissolved in 1x TAE or 1x TBE (section 2.16.3) buffer containing 0.5μg ethidium bromide/ml. Suitable amount of DNA sample was adjusted by diluting with water. 6x gel-loading buffer (section 2.16.3) was added to the DNA sample so that the final concentration of the loading buffer was 1x. The sample was loaded into the well and electrophoresis was performed at constant voltage of 80V in a gel tank containing 1x TAE or TBE (section 2.16.3). After electrophoresis was finished, DNA was visualized on a Spectroline Model TC-302 UV Transilluminator (302nm) and then photographed with a Polaroid MP-4 instant camera and Polaroid 667 instant film through a HOYA R(25A) red filter.

2.2.8 Restriction enzyme digestion of DNA

Digestion of DNA was carried out in a suitable volume depending on the amount of substrate. A suitable amount of restriction enzyme and the appropriate buffer was added to the substrate as suggested by the supplier. The reaction mixture was incubated at 37 °C for 2-3 hr. After digestion, the reaction mixture was analysed by agarose gel electrophoresis.

2.2.9 Purification of DNA fragment from agarose gel using Gene Clean® kit

The following method was suggested by the supplier. Either TAE or TBE was used as electrophoresis buffer. After agarose gel electrophoresis as stated in section 2.2.5, the gel was observed under UV illumination. The band containing the desired DNA was cut out from the agarose gel by a razor blade. The weight of the gel slice was measured and 3 vol of 6M NaI solution was added. If TBE had been used as buffer for electrophoresis, 2.5 vol of TBE modifier was added. The mixture was incubated at 55 °C until the gel slice was melted completely. Then 5-10μl of Glassmilk® silica matrix was added. The mixture was then kept on ice for 15 min with occasional shaking. The suspension was centrifuged in a microfuge at 12,000g (13,000rpm) for 1 min. The pellet was washed with 0.7ml of NEW WASH solution. The washing procedure was repeated for two more times and the NEW WASH solution was completely removed by a fine pipette tip. The washed pellet was resuspended in 5-10μl of water and incubated at 55 °C for 5 min to elute the DNA fragment. The supernatant containing the DNA was recovered by centrifugation in a microfuge at 12,000g (13,000rpm) for 1 min. The above DNA eluting procedure was repeated once and the total supernatant containing the DNA was pooled together and assayed by agarose gel electrophoresis to estimate the amount of DNA fragment recovered.

2.2.10 Ligation of insert into vector

The following method was modified from the suggestion of T4 ligase supplier, New England Biolabs Inc., which supplied us the T4 DNA ligase.

50ng of linearized vector, 20ng of insert, 1μl of 10x ligase buffer (0.5M Tris-HCl pH 7.8, 0.1M $MgCl_2$, 0.1M dithiothreitol, 10mM ATP, 250μg BSA) and 1μl of T4 DNA ligase (1Weiss unit) were mixed with water in a 1.5ml microcentrifuge tube to a final volume of 10μl. The reaction mixture was incubated overnight at 16 °C.

2.2.11 Rapid screening for the presence of plasmid by direct lysis of the bacterial colony

The following method was modified according to Sambrook (1989). After transformation, single colony was picked by sterilized toothpick and transferred to fresh LB agar plate with appropriate antibiotics. Bacterial colony was allowed to grow to 2-3mm in size. The bacterial cells were then transferred by a sterilized toothpick to the well of a 96-well plate which contained 20μl of the lysis buffer(50mM NaOH, 0.5% SDS, 5mM EDTA and 0.025% bromocresol green). The use of 96-well plate allowed the screening of large numbers of bacterial colonies at one time. The 96-well plate was sealed and incubated at 68 °C for 45 min. The mixture was then loaded to a 0.7% TBE agarose gel without the addition of ethidium bromide and the two ends of the gel were allowed just touching the running buffer. When the mixture had migrated into the gel, more TBE buffer was added to cover the gel. After electrophoresis, the gel was stained by submerged in 0.5μg/ml of ethidium bromide solution for 45 min. After staining, The gel was photographed under UV illumination as required.

2.2.12 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

All SDS-PAGE were run with the Mini-Protean II electrophoresis cell (BioRad). The cell was assembled according to the manufacturer's instructions.

Running gel, 12%, 15% and 20% were prepared as follow:

Gel percentage	12%	15%	20%
Water	1.6ml	1.1ml	0.6ml
30% acrylamide	2.0ml	2.5ml	3.0ml
1.5M Tris (pH8.8)	1.3ml	1.3ml	1.3ml
10% SDS	50µl	50µl	50µl
10% ammonium persulfate	50µl	50µl	50µl
TEMED	2µl	2µl	2µl

3ml of the running gel solution was pipetted into the gap between the glass plates. 200µl of isopropanol was added to keep the mixture out of atmospheric oxygen which affected the polymerization reaction and to remove air bubbles on the surface of the mixture. The gel was allowed to polymerize for 30 min. Then isopropanol was discarded and the gel was rinsed with water.

Stacking gel (3%) was prepared as follow:

Water	1.4ml
30% acrylamide	0.33ml
1.0 M Tris (pH6.8)	0.25ml
10% SDS	20µl
10% ammonium persulfate	20µl
TEMED	2µl

2ml of stacking gel mixture was pipetted into the gap between the glass plates and the comb was inserted. 15 min was allowed for the polymerization of the stacking gel.

The volume of protein samples were adjusted to 15µl with water and 15µl of 2x SDS gel loading buffer was added. The samples were denatured by boiling in water for 30 min and loaded to the wells. The gel was run at 30mA constant current until the bromophenol blue reached the bottom of the running gel. The Mini-gel was disassembled and the gel was removed for staining or to perform Western blot.

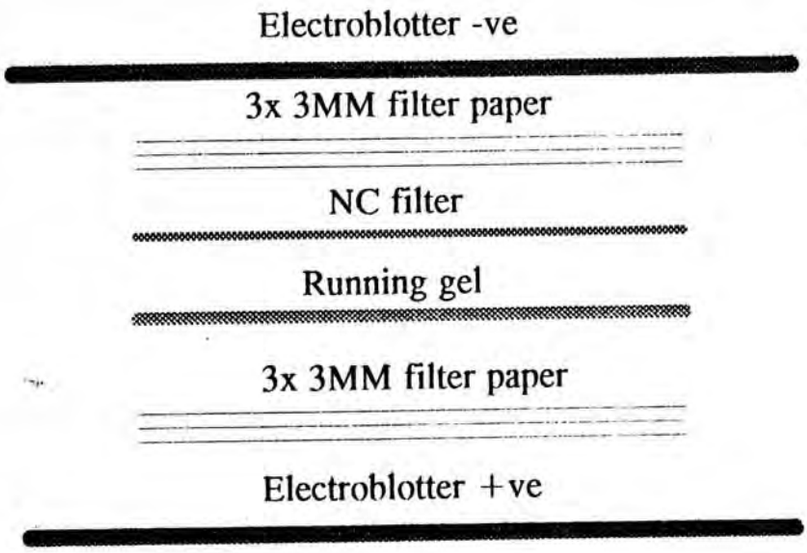
2.2.13 Staining of protein in polyacrylamide gel

After electrophoresis the gel was immersed in 100ml staining solution (0.15% (w/v) Coomassie Brilliant Blue R-250, 31.25% (v/v) ethanol, 10% (v/v) acetic acid) for 1 hour. The gel was destained in destaining solution (25%(v/v) ethanol, 8% (v/v) acetic acid) for overnight.

2.2.14 Western blot detection of TCS

After electrophoresis, the stacking gel was removed and the running gel was immersed in transfer buffer (section 2.16.5) for 5 min. The size of the running gel was measured. Six Whatman 3MM filter papers and one nitrocellulose filter (Amersham Hybond-C) of the same size as the running gel was cut out. The 3MM filter paper and nitrocellulose filter were wetted with

transfer buffer. The semi-dry electroblotter (BioRad) with the gel, 3MM filter paper and nitrocellulose filter were set up as follow:



A constant current of 0.8mA per cm^2 of nitro-cellulose filter was applied for 1 hr. ProtoBlot® Immunoscreening system from Promega was used for immunodetection of TCS. The following method was recommended by the supplier. After electroblotting, the nitrocellulose filter was immersed into 10ml of TBSTB (section 2.16.5) for 30 min. TBSTB was then discarded and 10ml of primary antibody (1:500 anti-TCS antibody) in TBSTB was added. The primary antibody was incubated for 2 hr at room temperature with continuous swirling. The filter was then washed repeatedly for 3 times with 20ml of TBST (section 2.15.5) at 3 min each. 10ml of anti-rabbit IgG or anti-mouse IgG antibody conjugated with alkaline phosphatase (1:7500 in TBSTB) was added and the filter was incubated with swirling at room temperature for 1 hr. The filter was then washed repeatedly for 3 times with 20ml of TBST at 3 min each. The filter was blotted dry with paper towels and incubated in color development solution (AP buffer) prepared as follow:

AP buffer (seection 2.15.5)	10ml
NBT substrate (50mg nitro blue tetrazolium /ml in 70% dimethylformamide)	66μl
BCIP substrate (50mg 5-bromo-4-chloro-3-indolyl phosphate /ml in 70% dimethylformamide)	33μl

The solution was swirled until color developed. When the color had developed to the desired intensity, the reaction was stopped by rinsing in water for several times. The filter was then air-dried for long term storage.

2.2.15 Autoradiography

Kodak X-OMAT AR diagnostic film was used for detection of DNA sequences. Films were exposed in a Kodak X-Omatic cassette at -70 °C for overnight to one week. Films were developed with 15% Kodak X-ray developer for 5 min, fixed with 15% Kodak X-ray fixer for 5 min and rinsed with tap water for 5 min.

2.3 Production of Recombinant Anti-TCS Fab Fragment

2.3.1 Cloning and screening of the recombinant anti-TCS Fab fragment

The cloning and screening of the recombinant monoclonal anti-TCS Fab fragments were done by Ms. X. B. Li, an academic visitor from China. The following is a brief summary. Ms. Li used an Immunozap™ Cloning Kit

from Stratacyte to clone the genes. Mouse was first immunized with TCS by Dr. C. C. Wong and the spleen was collected for extraction of mRNA. cDNA was synthesised using mRNA as template and PCR reaction for light chain and heavy chain genes. The PCR products were then cloned into the phage vector to produce a light chain library and a heavy chain library. Then the light chain library and the heavy chain library were ligated at random to produce a Fab phage library. ^{125}I labelled TCS was used to screen for positive Fab clones. Forty two positive clones were saved in phage stock and stored at 4°C for the later expression of Fab fragment.

2.3.2 Expression of recombinant anti-TCS Fab fragment

In vivo excision of phagemid plasmid from the phage vector

The following procedures were based on the Immunozap™ Cloning kit manual. Before the expression of Fab fragment, *in vivo* excision was done to allow cloned insert to be transformed to a convenience plasmid system. First of all, 200µl of XL1-Blue cells with OD_{600}= 0.5, 200µl of the phage stock that contained the Fab gene and 1µl of the R408 helper phage was mixed in a 1.5ml conical tube. The tube was then incubated at 37 °C for 15 min. Then 5ml of 2x YT medium was added and incubated for 3 hr at 37 °C with shaking. After incubation the tube was heated at 70 °C for 20 min, and then centrifuged for 5 min at 4000g in a bench top centrifuge. The supernatant was then transferred to a sterilized tube. 20µl of the supernatant was added with 200µl of XL1-Blue cell (OD_{600} = 0.5) in a 1.5ml microcentrifuge tube and incubated at 37 °C for 15 min. 1µl to 100µl of the mixture was plated onto LB plate containing 30µg/ml ampicillin and incubated overnight at 37 °C.

Overnight colony was streaked to new LB plate containing 30μg/ml ampicillin and glycerol stock was made for long term storage.

Expression of the Fab fragment

Overnight colony which contained the Fab gene was inoculated into 5ml of LBA broth (LB broth with 50μg ampicillin/ml). The culture was incubated overnight at 37 °C with vigorous shaking. The culture was then diluted 50-fold and transferred to a 500ml conical flask. The culture was to shaked continuously until the OD_{600} was about 0.6. Then IPTG was added to a final concentration of 1mM. The culture was then shaked continuously for 10 hr and the supernatant was collected by centrifugation at 4 °C for 20 min at 7,000g (8,000rpm Beckman J2-21 Centrifuge with rotor JA 20). The phagemid supernatant was then saved for further concentration.

The supernatant was concentrated by 500ml Amicon Ultra-filtration cell. 500ml of the supernatant was concentrated to 10ml and then dialysed against 1L of TBST overnight at 4 °C. Bovine serum albumin and sodium azide were added so that the final concentration were 1% and 0.03% respectively. The anti-TCS Fab fragment was then stored at 4 °C for later used.

2.4 Construction of Deletion Library of pET58210 by Transposon Mediated Mutagenesis

The procedures were based on the work of Sedgwick (1991). pET58210, a T7 expression vector with the TCS cDNA, was transformed

into MH1345 donor competent cells and spreaded on LBA agar plate with 50μg thymine/ml to select for positive colony. MH755 recipient cells were streaked into a selective LBS agar plate (LB agar plate with 100μg/ml streptomycin). A single colony of the donor cells with pET58210 was picked into 10ml of LBA broth with 50μg thymine/ml and grew at 37°C with vigorous shaking. A single colony of the recipient cells was picked into 10ml of LBS broth and grew at 37°C vigorous shaking. 0.1ml of the overnight cultures of donor cells and recipient cells were inoculated in 10ml fresh LB broth with 50μg thymine/ml, 50μg ampicillin/ml and 100μg streptomycin/ml respectively. The above culture was grown to log phase and was collected by centrifugation at 4,000g for 5 min at room temperature (5,000rpm Beckman J2-21 Centrifuge with rotor JA 20). The cell pellet was washed twice with fresh pre-warmed LB broth . The donor and recipient cells were resuspended together in 10ml of fresh LB broth without any antibiotics. The cell mating mixture was transferred to a 1L flask which had been pre-warmed to 37°C. After incubation at 37°C without shaking for 1 hr, the cell mixture was collected by centrifugation at 4,000g for 5 min at room temperature (5,000rpm Beckman J2-21 Centrifuge with rotor JA 20) and washed twice with fresh LB broth. 10-100μl cell mixture was spreaded onto selective plates which contained 50μg ampicillin/ml and 100μg streptomycin/ml. After overnight incubation at 37 °C, single colony was picked by toothpick and spreaded on a fresh LBAS plate. The plate was incubated overnight at 37 °C and saved for later PCR screening procedures.

2.5 PCR Insertion Mapping of the Transposon Inserted Clones

Plasmid DNA of the positive clones were prepared as described in section 2.1.1. Two PCRs for each clone were set up to map the insertion site of the transposon. For the first reaction, pET forward primer, Tn*1000* gamma and delta end primers were used. For the second reaction, primer B, Tn*1000* gamma and delta primers were used. The primer sequences were shown as follow:

1. pET forward primer
AACGGTTTCCCTCTAGAAAT

2. primer B
AACGCGATGTTGGAGGT

3. Tn*1000* delta end primer
AGGGGAACTGAGAGCTCTA

4. Tn*1000* gamma end primer
CAGCTACAACATACGAAAG

The following method was based on Sambrook *et al.* (1989) with modification. Plasmids from the positive clone were used as template for polymerization. Vent™ DNA polymerase and 10x amplification buffer were supplied by the New England Biolabs.

All PCRs were done in 25 µl total volume with light mineral oil as overlay In Temptronic temperature cycler machine. The reaction mixture contained 2.5µl of 10x amplification buffer, 4µl dNTP mix (1.25mM of dATP, dCTP, dGTP, dTTP), 2.5µl of pET forward (20µM) primer or primer B (20µM), 2.5µl of Tn1000 gamma primer and delta primer (20µM) and 0.5

U of Vent™ polymerase was added. Template (up to 0.5μg) was added to the reaction mixture. Water was added so that the final volume of the mixture was 25μl.

Amplification was carried out under the following cycles:

Cycle	Denaturation	Annealing	Polymerization
1st Cycle	3 minutes at 94°C	1 minute at 55°C	1 minute at 72°C
2nd to 34th Cycle	1 minute at 94°C	1 minute at 55°C	1 minute at 72°C
35th Cycle	1 minute at 94°C	1 minute at 55°C	6 minutes at 72°C

After amplification, 12μl of the amplification mixture was taken out and added with 3μl of 6x agarose gel loading buffer (section 2.16.3). The mixture was loaded to an agarose gel (section 2.2.7). After electrophoresis, photograph was taken to analyse for the positive clones.

2.6 DNA sequencing of positive clones after the PCR insertion mapping

Mutated pET58210 plasmid DNA with the transposon inserted within the TCS gene sequence were verified by DNA sequencing to locate the exact position of the transposon insertion site. Tn*1000* gamma end primer (sequence shown at section 2.5) was used as primer and the double stranded plasmid DNA as template.

2.6.1 DNA sequencing reaction

Sequencing reaction was carried out using Pharmacia T7 Sequencing kit. The following procedures were based on the manual provided by the supplier. Mutated plasmids DNA were prepared by Magic™ Miniprep (section 2.2.5). 2μg of plasmid DNA dissolved in 8μl of water was used as template. 2μl of 2M NaOH was added to the template and vortex gently to allow incubation at room temperature for 10 min. After denaturation of the template, 3μl of 3M sodium acetate (pH4.5) together with 7μl of H_2O was added to the mixture to neutralize the mixture. After mixing , 60μl of absolute ethanol was added to the mixture to precipitate the DNA. The mixture was stored at -70 °C for 30 min and the DNA was collected by centrifugation at 12,000g (13,000rpm) for 20 min at 4 °C in a microfuge. The pellet was washed with 80μl of ice-cool 70% ethanol and dried by SpeedVac. The dried pellet was then dissolved in 10μl of water and then 2 μl of annealing buffer and 5μg/ml primer solution were added to the template solution. The mixture was incubated at 65 °C for 5 min and 37 °C for 10 min and at room temperature for 5 min. 3μl of labelling mix, 1 μl of [α-^{35}S] dCTP (10mCi/ml, >1000Ci/mmol, Amersham SJ304) and 2μl of diluted T7 DNA polymerase (1.5units/μl, diluted with enzyme diluting buffer) were added to the reaction mixture. The mixture was incubated at room temperature for 5 min. During incubation, 2.5μl of A-, G-, C-, and T- mix short was added to four labelled fresh microfuge tubes which had been prewarmed at 37 °C. After incubation, 4.5μl of the reaction mixture was added to each of the four prewarmed sequencing mixes. The four tubes were incubated at 37 °C for 5 min. Then 5μl

of stop solution was added to each tube. The mixture was then stored at -20 °C for DNA sequencing electrophoresis.

2.6.2 DNA sequencing gel casting and electrophoresis

All sequencing gels were run with Sequi-Gen® Nucleic Acid Sequencing Cell. The glass plate and the IPC (Intergral Plate/Chamber which is a bound glass plate and buffer chamber) were cleaned with distilled water and ethanol thoroughly. Then the surfaces of the glass plates which contact the gel was wiped with repel-silane (dimethyldichlorosilane solution 2%(w/v) in 1,1,1-trichloroethane) using lint-free tissue paper. The unit was then assembled according to the manufacturer's instruction. 10ml of the acrylamide solution with the addition of 125µl 10% ammonium persulfate and 50µl TEMED was used to seal the bottom gap between the glass plate and the IPC. After the sealing gel had polymerized, the unit was laid in a tray so that the plate was inclined at an angle of about 20° . The remaining 90ml of acrylamide solution was added with 600µl 10% ammonium persulfate and 50µl TEMED. The gel was then poured into the gap between the glass plates and the IPC. The unit was then placed horizontally and a sharkstooth comb was inserted. The gel was allowed to polymerize for 1 hr.

When the polymerisation was completed, the sharkstooth comb was inserted with the teeth just touching the gel boundary. The unit was then assembled onto the gel tank and the lower and upper buffer reservoirs were filled with 1x TBE buffer. The gel was pre-run at 40W for 30 min. Before loading, the samples were heated at 80°C for 2 min. After the urea at the

wells had washed away, 3µl of sample was loaded into each well using sequencing pipette (Drummond). The gel was then run at constant power 40W to maintain a temperature of about 50°C. After the bromophenol blue tracking dye had just run to the bottom of the gel, electrophoresis was stopped and the sequencing cell was dissembled. The gel was then fixed in 10% v/v acetic acid for 10 min. Then the gel was blotted onto a sheet of 3MM Whatman filter paper. A piece of plastic wrap was used to cover the gel surface. The gel was dried in a gel dryer (BioRad) and exposed to an X-ray film (section 2.2.15).

2.7 Computer Analysis of the DNA Sequence

After DNA sequencing of the positive clones, the sequences were analysed using the computer programme DNASIS (Hitachi). The sequences were compared with the TCS gene sequence to find out the Tn*1000* insertion site and the orientation of the Tn*1000*. This information was then used to decide which subcloning procedures should be followed.

2.8 Subcloning of the Positive Clone that Have Been Located by DNA Sequencing

After DNA sequencing and computer analysis to find the exact location of the Tn*1000* insertion site, the deleted TCS gene sequence was subcloned by two methods depending on the orientation of the inserted Tn*1000*.

2.8.1 Subcloning of the mutated TCS gene sequence with the Tn*1000* delta end facing the N-terminal of the TCS gene sequence

Plasmid DNA with inserted Tn*1000* delta end orientated to the N-terminal of the TCS gene were prepared by Magic™ Miniprep (section 2.2.5). 5µg of the plasmid DNA was cut by *Bam*H I and *Nco* I (section 2.2.8). The reaction mixture was than loaded to a TBE agarose gel with Lamda *Hin*d III digested DNA and 123 bp ladder as markers. The size of the deleted TCS gene fragment visualized under UV after electrophoresis was cut and purified using GeneClean® kit (section 2.2.9).

5µg of pET8c was cut by *Bam*H I and *Nco* I. The reaction mixture was then loaded to a TAE agarose gel and after electrophoresis, the 4.6kb fragment was purified using GeneClean® kit and was finally dissolved in 20µl H_2O.

50ng of 4.6kb fragment of pET8c and 20ng of the deleted TCS gene fragment were ligated using T4 DNA Ligase (New England Biolabs) overnight at 16 °C (section 2.2.10). The ligation mixture (10µl) was added to 200µl of DH5α competent cells. Transformation was preformed as described in section 2.2.6.

The transformed colonies were screened by rapid lysis (section 2.2.11) for the presence of insert in pET8c. Higher molecular weight plasmid DNA as compared to pET8c was prepared by Magic™ Miniprep (section 2.2.4). The plasmid DNA was then analysed by restriction digestion with *Nco* I and

*Bam*H I followed by agarose gel electrophoresis to confirm the insertion of the TCS gene fragment into the pET8c vector.

2.8.2 Subcloning of mutated TCS gene sequence with the Tn*1000* gamma end facing the N-terminal of the TCS gene

Plasmid DNA with the inserted Tn*1000* gamma end orientated to the N-terminal of TCS gene was prepared by Magic™ Miniprep (section 2.2.5) and used as template for PCR reaction. A Tn*1000* gamma end mutagenic primer was made with the addition of a *Bam*H I site and was used as backward primer. The pET forward primer was used as forward primer (sequence shown at section 2.5). The primer sequence of the Tn*1000* gamma end mutagenic primer was shown as follow:

1. Tn*1000* gamma end mutagenic primer

*Bam*H I site

GCTATGGATCCACAGATGGGGAAACT

All PCRs were done in a total volume of 50µl with light mineral oil as overlay. The reaction mixture containing 5µl of 10x amplification buffer, 8µl dNTP mix (1.25mM of dATP, dCTP, dGTP, dTTP), 5µl of pET forward primer (20µM), 5µl of Tn1000 gamma end mutagenic primer (20µM) and 1U of Vent™ polymerase was added. Mutated pET58210 plasmid was used as template and was added to the reaction mixture. Water was added so that the final volume of the mixture was 50µl.

Amplification was carried out under the following cycles:

Cycle	Denaturation	Annealing	Polymerization
1st Cycle	3 minutes at 94°C	30 seconds at 55°C	30 seconds at 72°C
2nd to 34th Cycle	1 minute at 94°C	30 seconds at 55°C	30 seconds at 72°C
35th Cycle	1 minute at 94°C	30 seconds at 55°C	3 minutes at 72°C

After amplification, the DNA product was undergone phenol/ chloroform extraction and ethanol precipitation (section 2.2.1 & 2.2.2). The DNA product was then digested with *Nco*I and *Bam*H I (section 2.2.8). The digested mixture was then loaded to TAE gel with Lamda *Hind* III digested and 123 bp as markers. The molecular weight of the amplified mutated TCS gene sequence was calculated and the corresponding fragment visualized under UV after electrophoresis was cut and isolated by GeneClean® kit (section 2.2.9).

5μg of pET8c was cut by *Nco* I and *Bam*H I. The reaction mixture was then loaded to a TAE agarose gel and after electrophoresis, the 4.6kb fragment was purified using GeneClean® kit and was finally dissolved in 20μl H_2O.

50ng of 4.6kb fragment of pET8c and 20ng of the deleted TCS gene fragment were ligated using T4 DNA Ligase (New England Biolabs) overnight at 16 °C (section 2.2.10). The ligation mixture (10μl) was added to 200μl of DH5α competent cell. Transformation was preformed as described in section 2.2.6.

The transformed colonies were screened by rapid lysis (section 2.2.11) for the presence of insert in pET8c. Higher molecular weight plasmid DNA as compared to pET8c was prepared by Magic™ Miniprep (section 2.2.4). The plasmid was then analysed by restriction digestion with *Nco* I and *Bam*H I followed by agarose gel electrophoresis to confirm the insertion of the TCS gene fragment into the pET8c vector.

2.9 Construction of Deletion Mutants that Exclude the 21 Amino Acids in the N-terminal of TCS

Plasmid DNA of mutant 4 and 308 was prepared by Magic™ Miniprep (section 2.2.5) and used as template for PCR reaction. A forward deletion primer was made with the addition of a *Nde* I site was used as forward primer. A Tn*1000* gamma end mutagenic primer was made with the addition of a *Bam*H I site and was used as backward primer (sequence shown at section 2.7.2). The primer sequence of the forward deletion primer is shown as follow:

1. Forward deletion primer

Nde I site

GTTTTCCATATGAATCTGAGAAAAGCT

All PCRs were done in a total volume of 50µl with light mineral oil as overlay. The reaction mixture containing 5µl of 10x amplification buffer, 8µl dNTP mix (1.25mM of dATP, dCTP, dGTP, dTTP), 5µl of forward deletion primer, 5µl of Tn1000 gamma end mutagenic primer and 1U of Vent™ polymerase. Mutant plasmid was used as template and was added to the

reaction mixture. Water was added so that the final volume of the mixture was 50μl.

The amplification was carried out under the following cycles:

Cycle	Denaturation	Annealing	Polymerization
1st Cycle	3 minutes at 94°C	20 seconds at 55°C	20 seconds at 72°C
2nd to 34th Cycle	1 minute at 94°C	20 seconds at 55°C	20 seconds at 72°C
35th Cycle	1 minute at 94°C	20 seconds at 55°C	3 minutes at 72°C

After amplification, the DNA product was undergone phenol/ chloroform extraction and ethanol precipitation (section 2.2.1 & 2.2.2). The DNA product was then digested with *Nde*I and *Bam*H I (section 2.2.8). The digested mixture was then loaded to TAE gel with Lamda *Hin*d III digested and 123 bp ladder as markers. The molecular weight of the amplified mutated TCS gene sequence was calculated and the corresponding fragment visualized under UV after electrophoresis was cut and isolated by GeneClean® kit (section 2.2.9).

5μg of pET3c was cut by *Nde* I and *Bam*H I. The reaction mixture was then loaded to a TAE agarose gel and after electrophoresis, the 4.6kb fragment was purified using GeneClean® kit and was finally dissolved in 20μl H_2O.

50ng of 4.6kb fragment of pET3c and 20ng of the deleted TCS gene fragment were ligated using T4 DNA Ligase (New England Biolabs)

overnight at 16 °C (section 2.2.10). The ligation mixture (10μl) was added to 200μl of DH5α competent cell. Transformation was preformed as described in section 2.2.6.

The transformed colonies were screened by rapid lysis (section 2.2.11) for the presence of insert in pET3c. Higher molecular weight plasmid DNA as compared to pET3c was prepared by Magic™ Miniprep (section 2.2.4). The plasmid was then analysed by restriction digestion with *Nde* I and *Bam*H I followed by agarose gel electrophoresis to confirm the insertion of the TCS gene fragment into the pET3c vector.

2.10 Overexpression of Mutated TCS Protein with the T7 RNA Polymerase Driven Expression System

The subcloned plasmid DNA were then transformed into *E. coli* expression host BL21 (DE3, pLysS) to produce the deleted TCS protein by the T7 RNA polymerase driven expression system. Preparation of competent cells and transformation were preformed as stated in section 2.2.6.

A single colony of BL21 (DE3, pLysS) containing the deleted TCS gene was inoculated in 20ml of M9ZB (section2.15.1) with 100μg ampicillin/ml and 50μg chloramphenicol/ml in a 100ml conical flask. The culture was grown at 37°C overnight with vigorous shaking. The culture was shaked continuously at 37 °C for 4-5 hr until $O.D._{600}$ was about 0.8. Expression was then induced by adding isopropylthio-β-galactoside (IPTG) to a final concentration of 0.4mM. The culture was grown at 37°C for 3 more

hr. The cells were collected by centrifugation at 12,000g (13,000rpm) in a microfuge for 10 min at room temperature. The cells were then stored at -20°C for later assay.

2.11 Analysis of TCS Deletion Mutants by Western Blotting

The expressed cell pellet was used directly for Western blot analysis. The cell pellet was first added with 0.4ml of sample buffer (50mM Tris pH6.8, 2mM EDTA, 1% SDS, 1% mercaptoethanol, 8% glycerol, 0.025% bromophenol blue). The cells were then sonicated for 10 sec and denatured by boiling for 3 min. 15µl of the mixture was directly loaded to a 15% SDS-PAGE for electrophoresis (section 2.2.12). After electrophoresis, Western blot was performed (section 2.2.14). Recombinant monoclonal anti-TCS Fab fragment (section 2.3), classical monoclonal anti-TCS antibody and polyclonal anti-TCS antibody were used for the binding of deletion mutated TCS protein.

2.12 Preparation of Recombinant TCS Protein

Procedures of the work were based on Zhu *et al.* (1992). Expression vector containing recombinant TCS gene, pET58210, was transformed *into E. coli* expression host, BL21 (DE3, pLysS). Preparation of competent cells and transformation were performed as described in section 2.1.6. A single colony of BL21 (DE3, pLysS) containing pET58210 was inoculated in 10ml M9ZB medium with 100µg ampicillin/ml and 50µg chloramphenicol/ml. The culture was grown at 37°C overnight with vigorous shaking and then transferred to 1L M9ZB with the same amount of antibiotics. The culture was grown at

37°C with vigorous shaking until OD_{600} = 0.8 . The expression was then induced by adding isopropylthio-β-galactoside (IPTG) to a final concentration of 0.4mM. The culture was grown at 37°C for 3 more hr. The cells were collected by centrifugation at 4,000g (5,000rpm Beckman J2-21 Centrifuge rotor JA-14) for 10 min at 4 °C. After the supernatant was discarded, the weight of the cell pelleted was measured. The pellet was resuspended in 30 ml of 50mM NaH_2PO_4 (pH6.5) with 0.1mM phenylmethysulfonyl fluoride (PMSF) and 0.1% (v/v) β-mercaptoethanol. The suspension was sonicated for eight cycles (30 sec sonication and 30 sec cooling on ice) and was then centrifuged at 15,000rpm for 30 minutes at 4 °C. The supernatant was collected and dialysed against 50mM NaH_2PO_4, pH6.5 at 4 °C overnight.

After dialysis, the supernatant was applied to a CM Sepharose CL-6B column (2.5 x 26cm) which had equilibrated with buffer A (50mM NaH_2PO_4 ,pH6.5) and connected to the BioRad Econo System. The flow rate was kept at 2 ml/min. The column was washed with buffer A until OD_{280} of the eluant was less than 0.05. TCS was eluted by applying a 120ml 0 - 0.5 M NaCl gradient at a flow rate of 2ml/min. The peak was collected and dialysed against 2L buffer A overnight at 4°C. The column was then washed with high salt buffer and equilibrated with buffer A again. The dialysed solution was then applied to the CM Sepharose CL-6B column again with a flow rate of 2ml/min. TCS was eluted by applying a 120ml 0 - 0.3 M NaCl gradient at a flow rate of 2ml/min. The peak was collected and dialysed against 2L distilled water. The purified TCS was then snap-freezed in liquid nitrogen and lyophilized. The lyophilized protein was then stored at 4°C for later use.

2.13 Proteolytic and Chemical Cleavage of Recombinant TCS

Three proteolytic enzymes and one chemical agent were used to cut the recombinant TCS into smaller peptides for epitope detection using Western blotting analysis.

Proteolytic enzyme cleavage

Alkaline protease (Promega), Endoprotease Lys-C (Promega) and Glu-C (Promega) were used for the cleavage of recombinant TCS protein. The enzymatic cleavage reactions were done in a microcentrifuge tube with 0.1M Tris-HCl pH8.0 and 0.1% SDS as reaction buffer. Protease was added so that protease : protein ratio was 1%. The digestion mixtures were incubated at 37°C for 90 min. The digested products were then analysed in 16.5% high resolution SDS-polyacrylamide gel (section 2.12).

Chemical cleavage

Cyanogen bromide was used for chemical cleavage of recombinant TCS protein. 1mg of cyanogen bromide and 1mg of recombinant TCS protein were dissolved in 300µl of 50% Trifluoroacetic acid. The digestion mixture was incubated at room temperature for 6 hr with constant agitation. After incubation, the reaction was stopped by lyophilization. The lyophilized product was then analysed by 16.5% high resolution SDS-polyacrylamide gel (section 2.12).

2.14 Tricine-SDS-16.5% Polyacrylamide Gel Electrophoresis

The following method was developed by Schägger and von Jagow (1987).

Stock solution

The stock solutions for gel electrophoresis are listed in the following tables:

Buffer	Tris (M)	Tricine (M)	pH	SDS (%)
Anode	0.2	-	8.9[a]	-
Cathode	0.1	0.1	8.25[b]	0.1
Gel buffer	3.0	-	8.45[a]	0.3

[a] Adjusted with HCl

[b] No correction of the pH, which was around 8.25

Acrylamide-bisacrylamide mixture	Percentage acrylamide (w/v)	Percentage bisacrylamide (w/v)
49.5% T, 3% C	48	1.5
49.5% T, 6% C	46.5	3.0

T = total percentage of acrylamide and bisacrylamide

C = crosslink percentage of the acrylamide gel

Preparation of glass plates for gel casting

The surfaces of a pair of glass plates (20 x 15.5cm, 17 x 15.5cm) were cleaned with distilled water and ethanol thoroughly. Three 0.7mm thick spacers were placed in between. The plate sandwich was fixed by using six

metal clips. Finally the three edges of the sandwich were sealed with 1% agarose.

Gel casting

The compositions of separating, spacer and stacking gel are shown in the following table:

	Stacking gel 4% T, 3% C	Spacer gel 10% T, 3%C	Separating gel 16.5% T, 6% C
49.5% T, 3% C solution	1 ml	6.1 ml	-
49.5% T, 6% C solution	-	-	10 ml
Gel buffer	3.1 ml	10 ml	10 ml
Glycerol	-	-	4.3ml
Added water to a final volume of	12.5 ml	30 ml	30 ml

Degas of the gel solutions were not required

The separating gel and the spacer gel were polymerized together in the glass plate. Because the presence of glycerol in the separating gel, the two gel solutions did not mix together in the process of adding the gel solution. With the addition of 150µl 10% ammonium persulfate and 15µl TEMED, the separating gel solution was first poured between the gap of the glass plate sandwich prepared in the previous step. Then the spacer gel solution with the addition of 100µl of 10% ammonium persulfate and 10µl TEMED was poured slowly between the gap of the glass plate sandwich. Then 1ml of isopropanol was added to keep the solution from atmospheric oxygen and to

remove air bubbles. The separating gel and spacer gel were allowed to polymerized together for 30 min. After the separating and spacer gel had polymerize, the isopropanol layer was removed. The stacking gel with the addition of 100µl 10% ammonium persulfate and 10µl TEMED was poured and the comb was inserted to the gap between the glass plate.

Electrophoresis

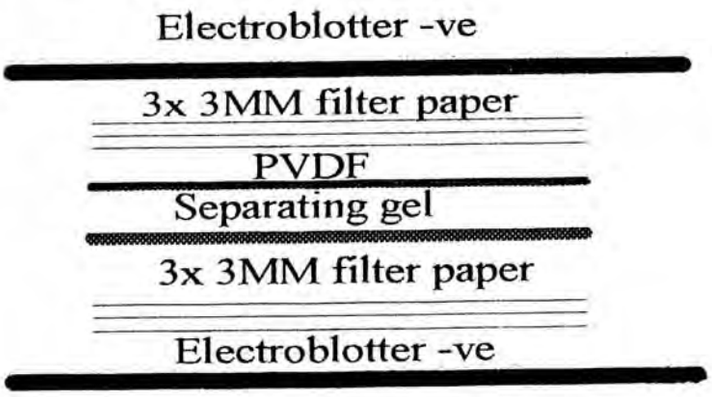
When the polymerization was completed, the comb was removed and the wells were washed thoroughly with water to remove any trace of unpolymerized monomer. The gel plate sandwich was then assembled onto the gel tank. The upper buffer reservoirs were filled with cathode buffer and the lower buffer reservoir was filled with anode buffer. The protein samples with the addition of 2x SDS-PAGE sample buffer (section 2.15.4) and denatured at 100°C for 3 min were loaded into wells. Electrophoresis was started at 30V constant voltage for 1 hr and then 15mA constant current for 24 hr. After electrophoresis, the glass plates were dissembled and the gel was used for staining of Western blotting.

Fixing, staining and destaining

The protein bands were fixed in a solution containing 50% methanol and 10% acetic acid for 30 min before they were stained with 0.025% Coomassie Brilliant Blue R-250 in 10% acetic acid for 1-2 hr. The gel was then destained in 10% acetic acid for 2 hr.

2.15 Electroblotting Using Polyvinylidene Difluoride Membrane (PVDF) for N-terminal Determination

After electrophoresis (section 2.12), the stacking and spacer gels were removed and immersed in electrobuffer (200ml 10x CAPS (section 2.15.7) + 200ml methanol + 1600ml H_2O). The size of the separating gel was measured. Six Whatman 3MM filter papers and one PVDF (Millipore Immoblin-P PVDF) of the same size as the running gel was cut out. The 3MM filter papers and PVDF membrane were wetted with electrobuffer. The semi-dry electroblotter (BioRad) with the gel, 3MM filter papers and PVDF membrane were set up as follow:



A constant current of 0.8mA per cm^2 of PVDF membrane was applied for 1 hr. After blotting, the PVDF membrane was removed and rinsed with deionized water. The PVDF membrane was then stained with the staining solution (0.15% (w/v) Coomassie Brilliant Blue R-250, 31.25% (w/v) ethanol, 10% (v/v) acetic acid) for 5 min. The PVDF membrane was then destained in destaining solution (25% (v/v) ethanol, 8% (v/v) acetic acid) for 1 hr. The PVDF was then blotted dry and the interested protein band was cut out for N-terminal determination.

2.16 Reagents and Buffers

2.16.1 Media for bacterial culture

LB (Luria-Bertani medium)

10g bacto-tryptone

5g bacto-yeast extract

10g NaCl

The solution was adjusted to pH7 with 10M NaOH and the volume was made up to 1 litre with water. The medium was sterilized by autoclaving for 20 minutes at 15 lb/sq. in. pressure on liquid cycle.

ϕb medium

5g bacto-yeast extract

20g bacto-tryptone

10.2g $MgSO_4.7H_2O$

The volume was made up to 1 litre with water and the medium was sterilized by autoclaving for 20 minutes at 15 lb./sq.in. pressure on liquid cycle.

M9ZB medium

1g NH_4Cl

3g KH_2PO_4

15.2g $Na_2PO_4.12H_2O$

10g Tryptone

5g NaCl

The volume was made up to 1 litre with water. The medium was sterilized by autoclaving for 20 minutes at 15 lb./sq.in pressure on liquid cycle. 20 ml of 20% glucose and 1 ml of $MgSO_4$ was added to the medium just before use.

YT medium

10g NaCl

10g bacto-yeast extract

10g bacto-tryptone

The volume was made up to 1 litre with water and the medium was sterilized by autoclaving for 20 minutes at 15 lb./sq.in. pressure on liquid cycle.

Media containing agar

Liquid media were prepared according to the recipes given above. Just before autoclave, 15g of bacto-agar was added to 1 liter liquid medium.

2.16.2 Reagents for preparation of plasmid DNA

P1 (stored at 4°C)

5mM Tris-Cl (pH8.0)

10mM EDTA

100µg/ml RNase A

P2 (stored at RT)

0.2M NaOH

1% SDS

P3 (stored at RT)

2.55 M potassium acetate (pH4.8)

2.16.3 DNA electrophoresis buffers

TAE (Tris-acetate), 1x

40mM Tris-acetate

1mM EDTA

TBE (Tris-borate), 1x

90mM Tris-borate

2mM EDTA

6x agarose gel loading buffer

0.25% bromophenol blue

40% (w/v) sucrose in water

2.16.4 Reagents for SDS-PAGE

2x SDS gel-loading buffer

100mM Tris-Cl pH6.8

200mM β-mercaptoethanol

4% SDS

0.2% bromophenol blue

20% glycerol

β-mercaptoethanol was added just before the gel-loading buffer was used.

1x SDS-PAGE buffer

25mM Tris-Cl

0.1% SDS

192mM glycine

Staining solution

0.144% (w/v) Coomassie Brilliant Blue R-250

31.25% (v/v) ethanol

10% (v/v) acetic acid

Destaining solution

25% (v/v) ethanol

8% (v/v) acetic acid

2.16.5 Reagents for Western blot

Transfer buffer

48mM Tris-Cl

39mM glycine

1.3mM SDS

20% (v/v) methanol

TBST

10mM Tris-Cl pH8.0

150mM NaCl

0.05% Tween 20

TBSTB

TBST containing 0.3% Bovine Serum Albumin

AP buffer

100mM Tris-Cl pH 9.5

100mM NaCl

5mM $MgCl_2$

BCIP

5-Bromo-4-chloro-3-indolylphosphate

(50mg/ml in 70% dimethylformamide)

NBT

Nitroblue tetrazolium

(50mg/ml in 70% dimethylformamide)

2.16.6 Reagents for DNA sequencing

40% acrylamide

38g Acrylamide

2g N',N'-methylene-bisacrylamide

The mixture was dissolved in water. The solution was made up to 100ml after the solute had dissolved. 5g of amberlite ion exchange resin (Sigma) was added and the mixture was stirred at room temperature for 30 min for the adsorption of metal ions that might inhibit the polymerization of acrylamide. The resin was then removed by filtration through a 3MM filter paper. The acrylamide solution was stored at 4°C in a blue cap bottle.

2.16.7 Reagents for electroblotting using PVDF

10x CAPS buffer

10x stock (100mM, pH11) solution was prepared by dissolving 22.13g of 3-[cyclohexlamino]-1-propane-sulphonic acid in 990ml of de-ionized water. The solution was then titrated with 2M NaOH to pH11 and de-ionized water was added to make a final volume of 1 litre. The solution was stored at room temperature.

Chapter Three

The Study of Antigenic Determinants of TCS by Transposon Mediated Deletion Mutagenesis

3.1 Introduction

In order to study the antigenic determinants of TCS, deletion mutants were produced by transposon mediated mutagenesis. The approach to construct deletion library using bacterial Tn*1000* transposon is outlined diagramatically in Fig. 3.1.

Tn*1000* is a natural part of the *E. coli* F sex factor and transposes into other plasmids co-habiting the same male bacterium at low frequency. During transposition, Tn*1000* is duplicated in a transitory linkage of F factor joined with the target plasmid. Once in the female or recipient, this co-integrate molecule undergoes a site-specific recombination reaction to restore F factor and release a target plasmid with a transposon insertion (Sedgwick *et al.*, 1991).

After transposition, Tn*1000* would be inserted randomly into the plasmid containing the TCS gene sequence. If the Tn*1000* was inserted within the TCS coding sequence, premature translation termination would occur. This is because both the gamma and delta ends of the Tn1000 possess the translation termination codons in three reading frame (Fig.3.2). The codons marked with * are the corresponding stop codons for premature translation termination. The insertion of transposon into the TCS coding sequence would therefore result in the production of a series

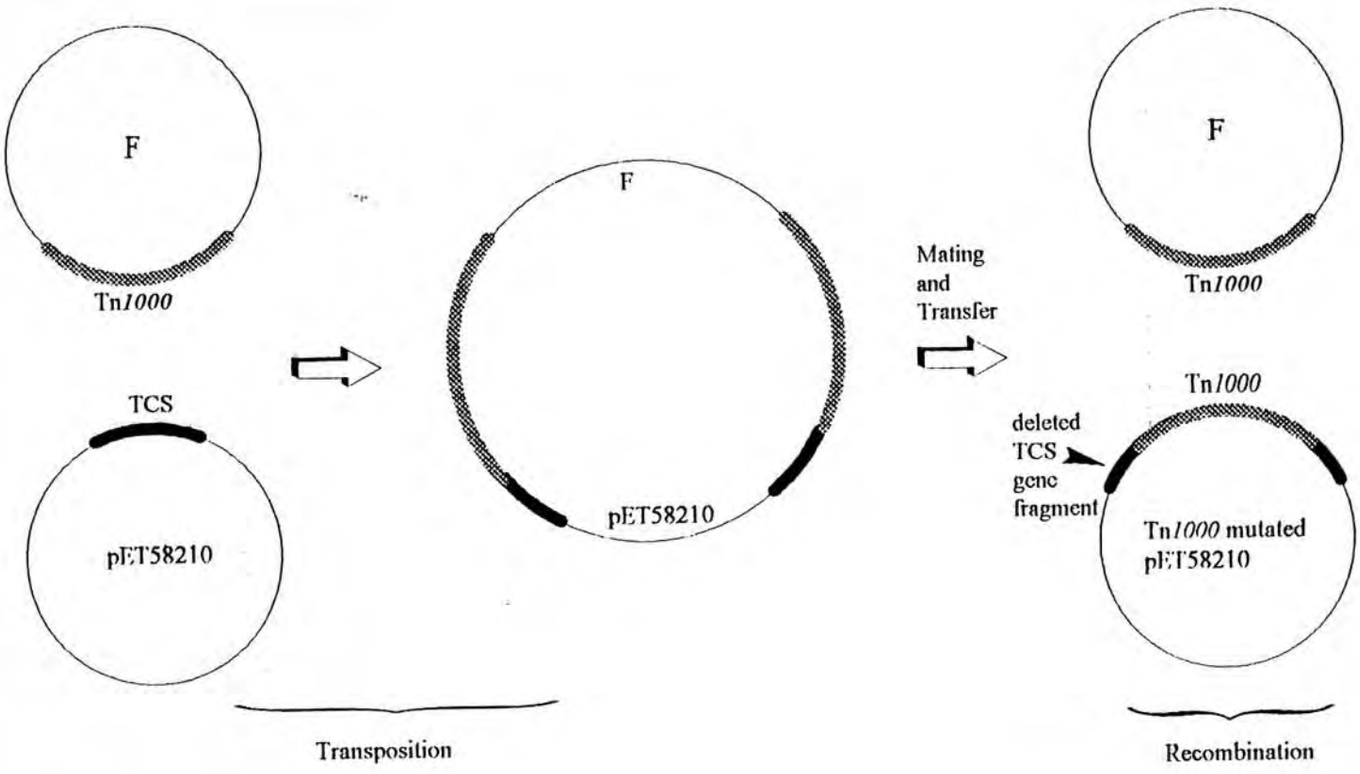


Fig. 3.1 Transposition mechanism for Tn*1000* mutagenesis of plasmid DNA. Tn*1000* on sex factor F transposes into the target plasmid pET58210 which contains the TCS gene sequence. The transposition results in the formation of a co-integrate. The co-intergrate is transferred to a female cell where site-specific recombination in Tn*1000* will result in a restored F and leaving the target plasmid with an inserted Tn*1000*.

translational stop codons occur at both end

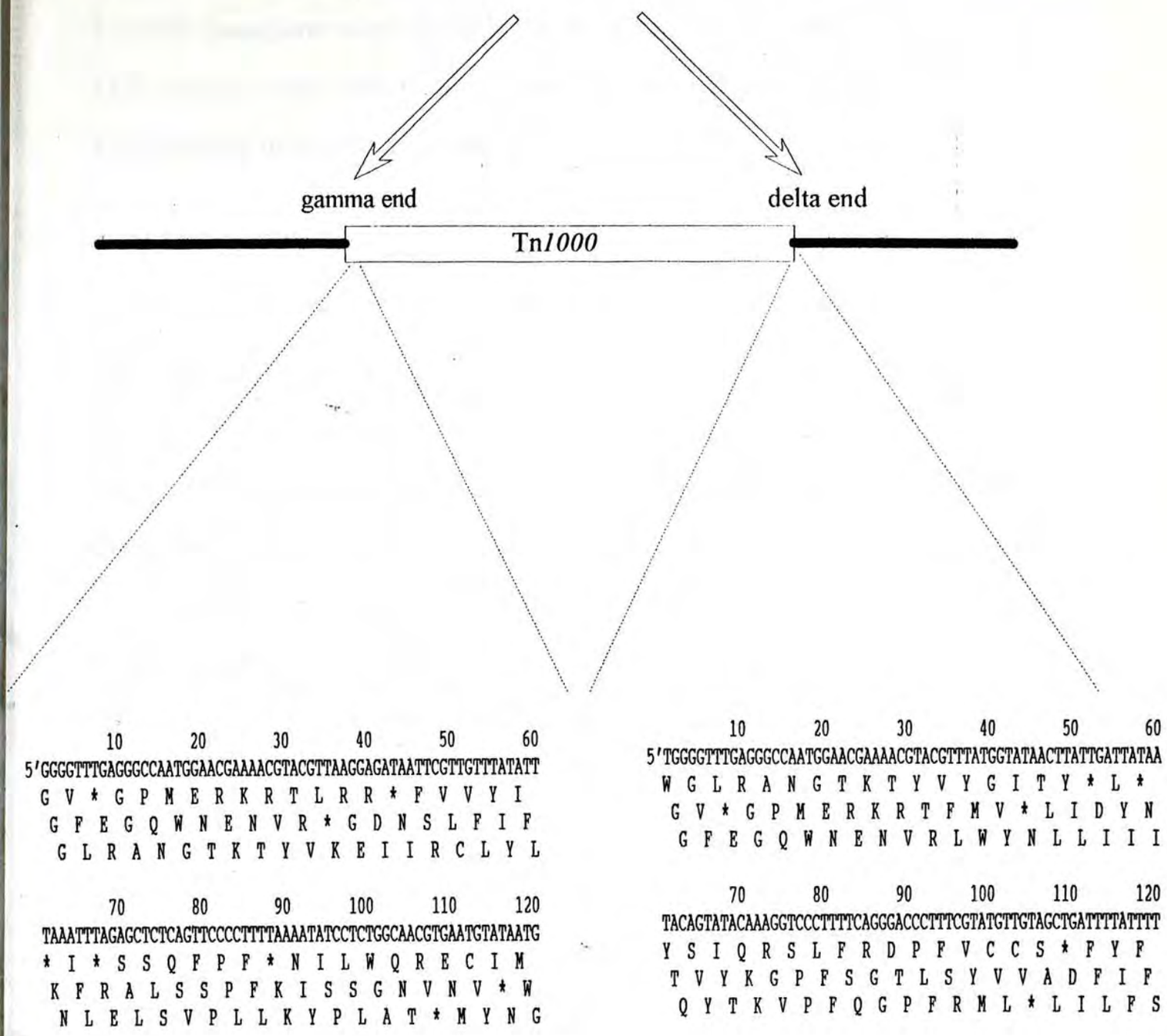


Fig.3.2. Diagram showing the position of the translational stop codons at Tn *1000*.

of TCS truncated protein with different length. The precise location of the Tn1000 insertions were found out by DNA sequencing. The truncated TCS protein was then analysed by Western Blotting. Shorter truncated TCS protein would be recognized by fewer antibodies. Together with the precise location of the Tn*1000* insertion sites, a detail mapping of the epitopes of TCS can be generated.

3.2 Results

3.2.1 Construction of deletion library of pET58210 by transposon mediated mutagenesis

To perform transposon Tn*1000* mediated mutagenesis, the first step is to transform the donor strain, MH1345, with the target plasmid, pET58210 which contains the TCS gene sequence. MH1345 has a *recA1* mutation to improve plasmid stability. The *rpsL*$^{+}$ and *deoC* markers of MH1345 confer streptomycin sensitivity and thymine dependency respectively and were used to counter-select against the donor after mating. MH755 was used as recipient cells which has a *rpsL* mutation and hence confers resistance to streptomycin. After bacterial mating the recipient cells were selected by streptomycin and ampicillin. The presence of streptomycin and lack of thymine prevents the growth of donors after mating. Since the only means of target plasmid transfer is via a transposition event, all surviving recipients on the selective plates contain transposed target plasmids. The plate which had spreaded with 10-100μl of mating samples were found to contain about 10-1000 single colonies on the selective plates. As shown in Fig.3.3, the size of the plasmids isolated from the recipient cells were larger than pET58210 which showed that a

Fig. 3.3. Lanes (1,2): plasmid DNA isolated after the mating. Lane (3): pET58210. Lane (4): λ *Hin*d III marker.

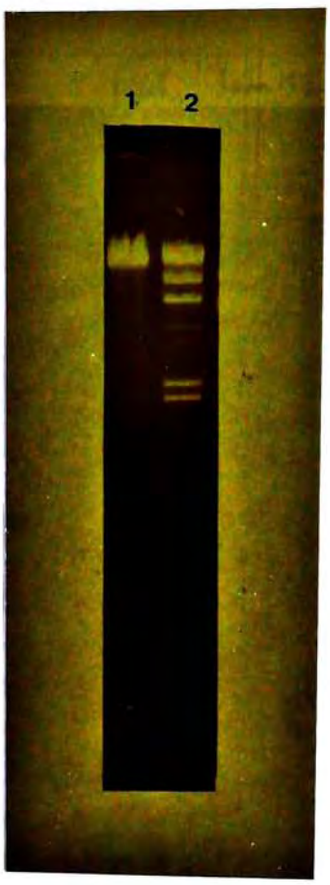


Fig. 3.4. Lane (1):, plasmid DNA isolated after mating was cut with *Kpn* I, Lane (2): λ *Hin*d III marker.

Tn1000 had inserted into the pET58210 through the transposition process. Because the pET58210 is 5.4kb and the Tn*1000* is 5.9kb, the mutated plasmid should be 11.3kb. In Fig.3.4, the mutated plasmid was cut with *KpnI* which has only one cutting site in Tn*1000* and a fragment of about 11.3kb was observed. This shows that the plasmid DNA isolated from the recipient cell was the result of Tn*1000* inserted into pET58210. About 300 independent recipient colonies were isolated and they were then undergone PCR insertion mapping to find out whether the Tn*1000* was inserted within the TCS gene sequence.

3.2.2 PCR insertion mapping of the transposon inserted clones

Because the TCS gene sequence is 0.747kb and the pET58210 was 5.4kb in size, theoretically only about 10% (0.747/5.4) of the mutated plasmid will possess a Tn*1000* inserted within the TCS gene sequence. Therefore PCR insertion mapping was done to screen for these 10% mutants. The methods of PCR insertion mapping was described in section 2.5. The strategy of the PCR insertion mapping was shown in Fig.3.5. If Tn1000 was inserted within the TCS gene sequence, the first reaction will result in the production of the N-terminal TCS gene fragment and the second reaction will result in the production of the C-terminal TCS gene fragment. The sum of the length of the two fragments will equal approximately to that of the whole TCS gene fragment. The use of both Tn*1000* gamma and delta end primers in the same reaction is because Tn*1000* can insert in either orientation. Fig.3.6 is a typical result of the PCR insertion mapping. Lanes 3&4 and lanes 1&2 have no reaction product which means that Tn*1000* was inserted outside the TCS gene sequence. In lanes 11&12, only one reaction product appeared which

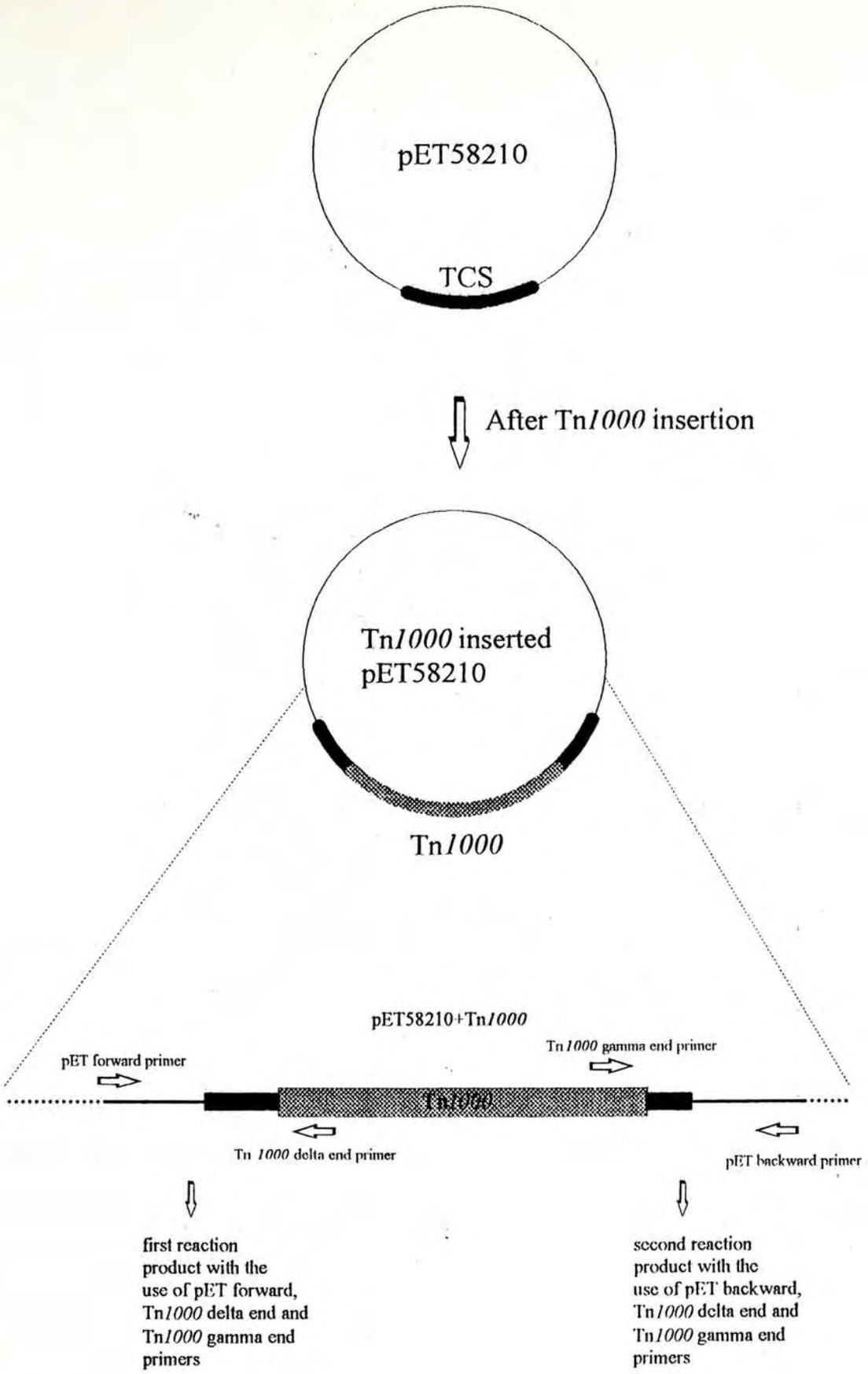


Fig.3.5 PCR insertion mapping for locating the site of Tn*1000* insertion.

Fig. 3.6. Typical results of PCR insertion mapping. Lanes 1, 3, 5 and 11 are the first PCR products of 4 different mutants. Lanes 2, 4, 6 and 12 are the second PCR products of 4 different mutants. Lanes (1&2, 3&4, 11&12): Tn1000 inserted outside TCS gene sequence. Lane (5&6): Tn*1000* inserted within TCS gene sequence. Lane (8): 123 base pairs ladder marker. Lane (9): λ *Hind III* marker.

means that Tn*1000* was inserted near the C-terminal of the TCS gene sequence as confirmed by DNA sequencing. In lanes 5&6, two reaction products were observed which shows that the Tn*1000* was inserted within the TCS gene sequence. Three hundred clones from section 3.2.1 were screened by PCR insertion mapping and 15 clones showed positive result in PCR insertion mapping reaction. These mutants were than verified by DNA sequencing to locate the exact position of the transposon insertion site.

3.2.3 DNA sequencing of positive clones after the PCR insertion mapping.

The precise site of transposon insertion was determined by DNA sequencing with primers complementary to unique sequence in the gamma end of Tn*1000*. The Tn*1000* insertion can be recognized because the gamma end of Tn*1000* is characterized by a sequence of inverted repeats. A typical sequencing gel with four clones is shown in Fig.3.7. The polymerase initially copies the transposon sequence up to AAACCCC which is the terminal inverted repeats of Tn*1000* and subsequently the TCS gene sequence at the transposon insertion position. For clone 86, the TCS sequence is TTGTAGTAAAAC.... For clone 163, the TCS sequence is TTATTTTGCCTG.... For clone 243, the TCS sequence is TTTGAAGCCTTT... and for clone 277, the TCS sequence is GAATATGAAGCG.... The 15 clones that were isolated by PCR insertion mapping were all sequenced and the DNA were analysed by a computer programme.

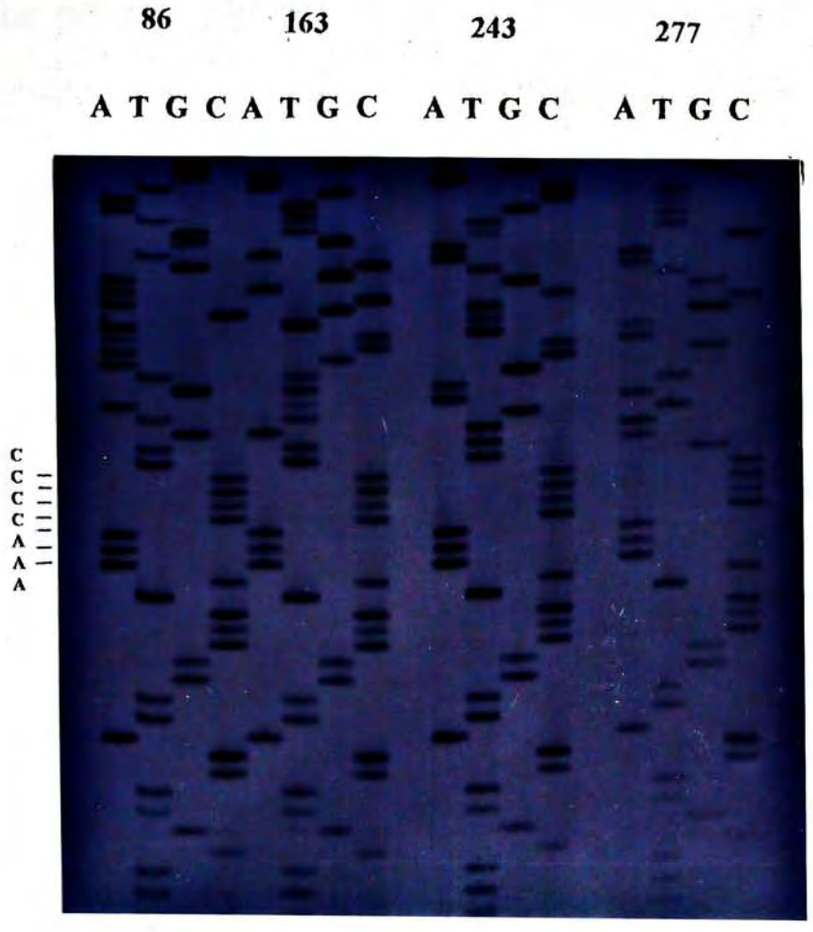


Fig. 3.7. Sequencing results of mutants 86, 163, 243 and 277. The polymerase initially copies the Tn*1000* sequence up to AAACCCC and subsequently the TCS cDNA sequence at the transposon insertion site position.

3.2.4 Computer analysis of the DNA sequence

Sequences of the positive clones were analysed by the computer programme DNASIS (Hitachi). The analysis aims at comparing the sequence of the positive clone with the TCS gene sequence and hence found out the exact location of the Tn*1000* insertion site and its insertion orientation. Since the Tn*1000* can insert in either orientation, therefore the determination of the orientation of the Tn*1000* is very important for two reasons.

Firstly, the insertion of the Tn*1000* at the TCS sequence will result in the duplication of five bases at the target DNA, and when one reads the sequencing ladder, correction of the insertion should be made before finding the Tn*1000* insertion site .

Secondly, the orientation of the Tn*1000* insertion will determine which subcloning procedures should be followed (section 3.26). After computer analysis, the exact location of the Tn*1000* insertion site and the orientation of the inserted Tn*1000* will be known. The insertion position of the Tn*1000* of the 15 clones is shown in Fig.3.8.

3.2.5 Expression of mutated plasmids with Tn*1000*

Mutated plasmid with Tn*1000* inserted within the TCS gene sequence was expressed in the T7 RNA polymerase driven expression system to produce the mutated protein. Fig. 3.9 shows the expression products. Unexpectedly, expression of the mutated TCS with Tn*1000* resulted in the production of natural TCS. Therefore all mutated plasmids

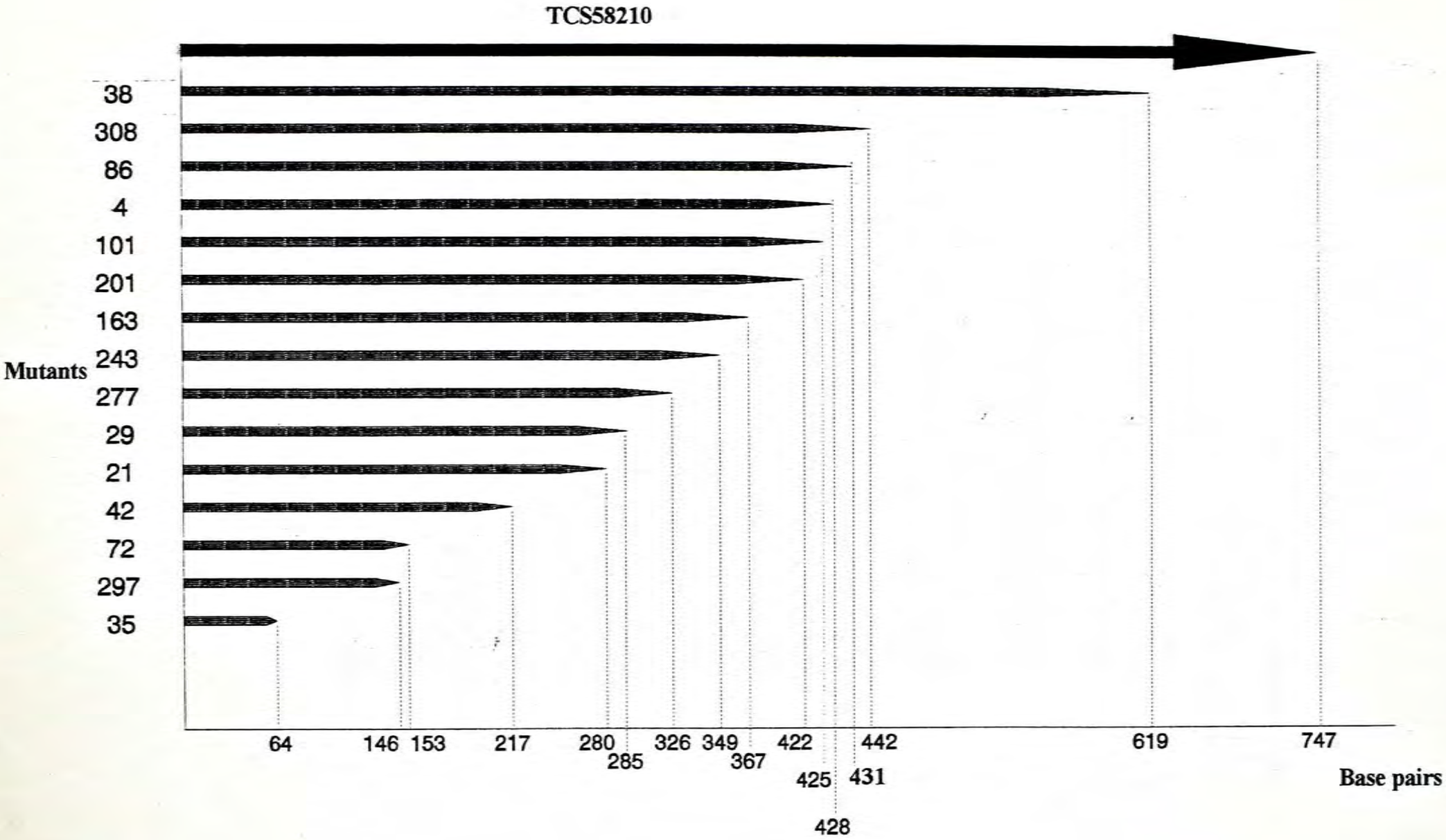


Fig.3.8 Mutants of TCS that were produced by transposon mediated mutagenesis.

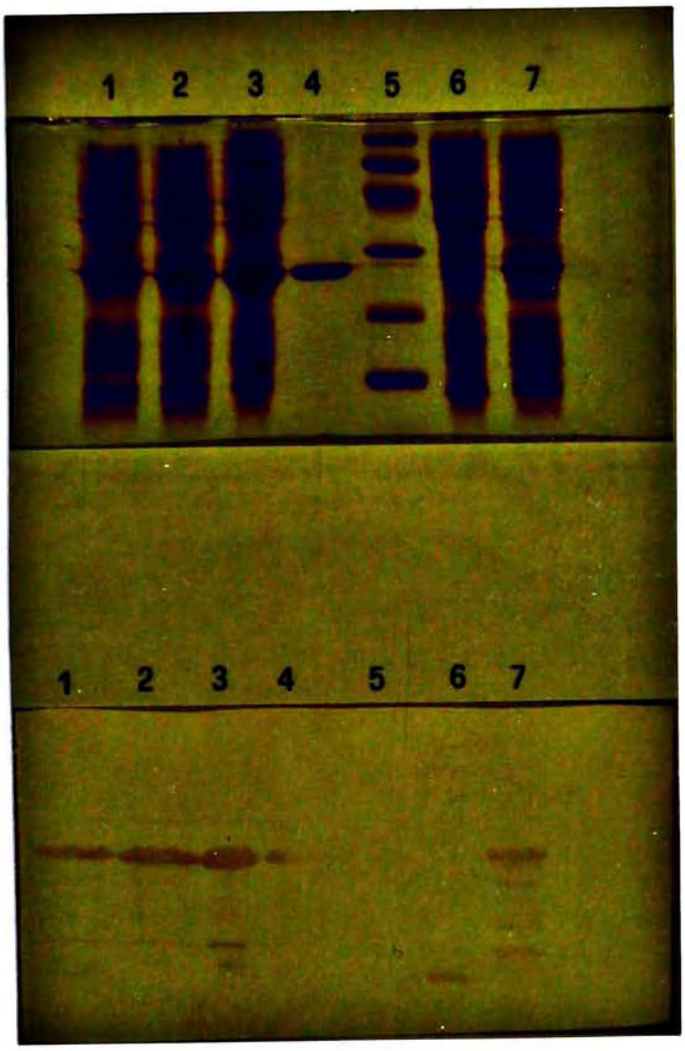


Fig. 3.9. The expressed products analysed by SDS-PAGE and Western blotting. Lanes (1,2): mutants with Tn*1000* 29, 38 respectively. Lane (3): normal expressed TCS. Lane (4): purified TCS. Lane (5): molecular weight marker. Lanes (6,7): subcloned mutant 29,38, respectively.

were subcloned to eliminate the effect of Tn*1000* on the expression of truncated TCS protein.

3.2.6 Subcloning of the positive clones that have been characterized by DNA sequencing

As we suspect that Tn1000 may have an adverse effect on the expression of truncated TCS, subcloning procedures were followed to isolate the mutated TCS gene fragment. Because the Tn*1000* can insert in either orientation, two subcloning methods were followed depending on the Tn*1000* inserted orientation in the mutated clones.

The methodology of the first subcloning method is outlined in section 2.8.1 and the strategy of subcloning is shown in Fig.3.10. If the delta end of the transposon is orientated in the N-terminal of TCS gene sequence, the deleted TCS gene sequence can be isolated directly by cutting with *Nco I* and *BamH I*.

The methodology of the second subcloning method is outlined in section 2.8.2 and the strategy of subcloning is shown in Fig. 3.11. A Tn*1000* gamma end mutagenic primer with the addition of a *Bam*H I site was made (sequence shown at section 2.7.2). Together with the pET forward primer, the deleted TCS gene fragment was amplified by PCR.

Among the 15 Tn*1000* inserted plasmids, eight of them were subcloned by the first method and seven of them were subcloned by the second method.

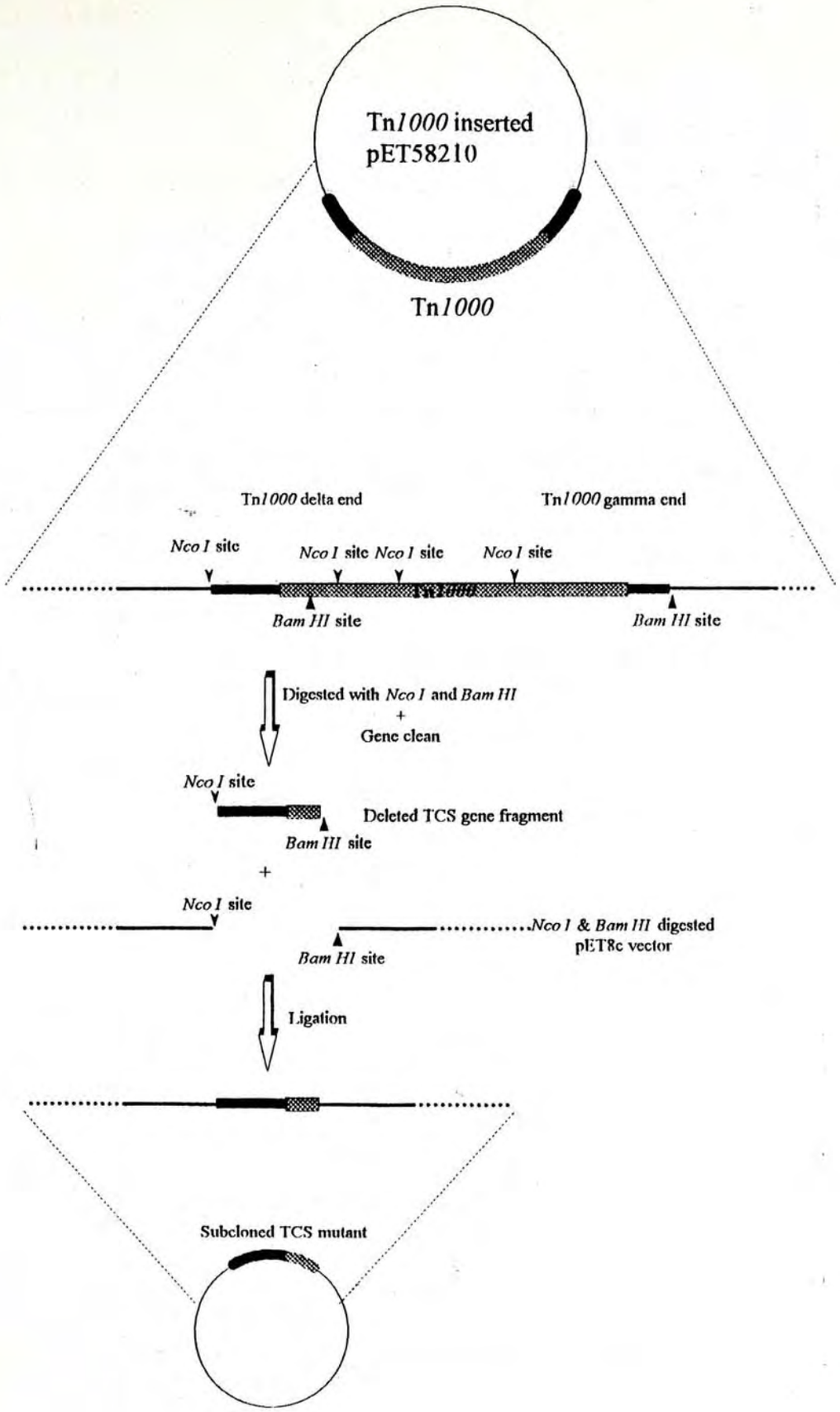


Fig.3.10 Subcloning of the deleted TCS gene fragment at the Tn*1000* delta end by restriction enzyme.

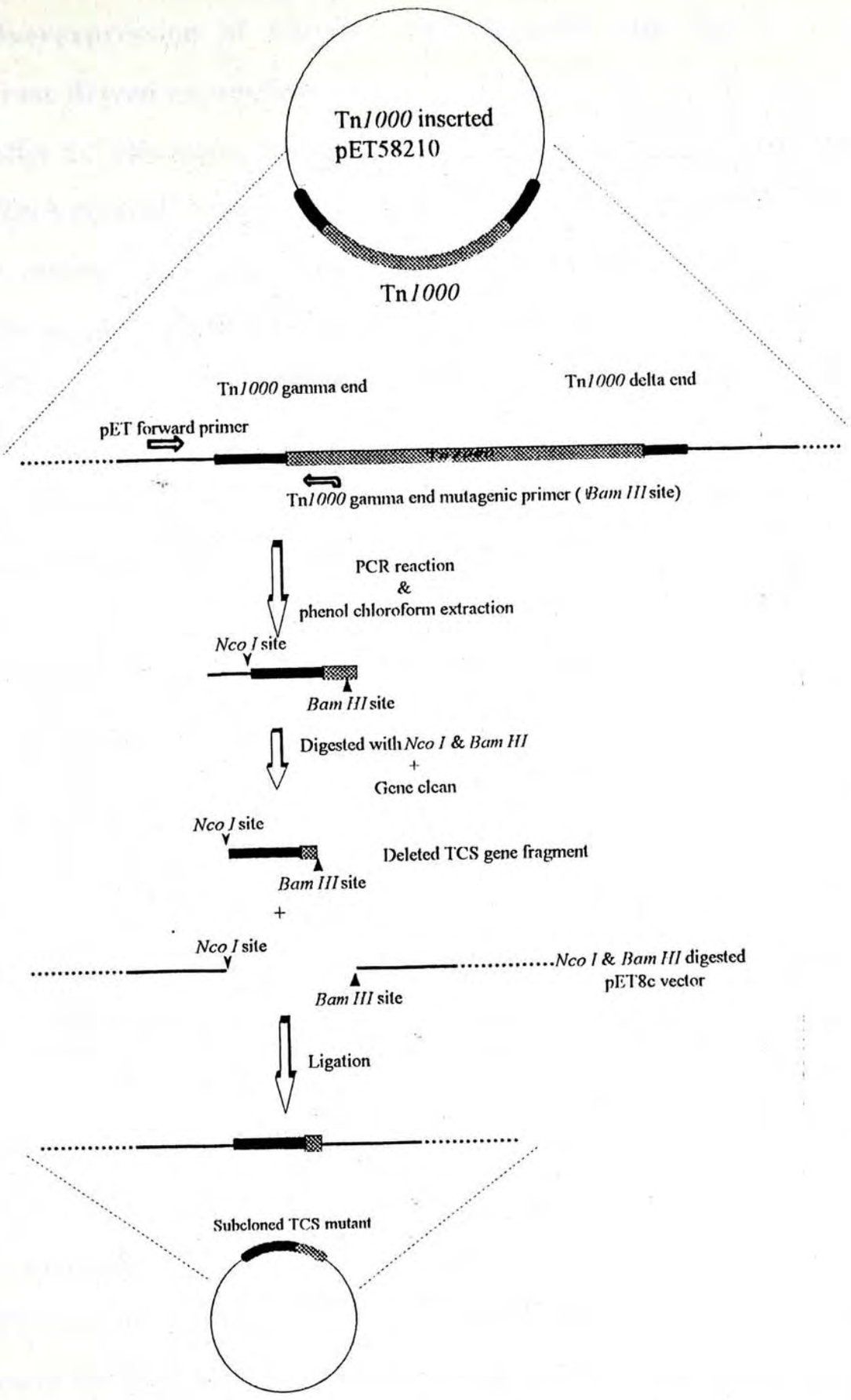


Fig.3.11 Subcloning of the deleted TCS gene fragment at the Tn *1000* gamma end by PCR.

3.2.7 Overexpression of mutated TCS protein with the T7 RNA polymerase driven expression system

After the subcloning procedures, mutated proteins were produced in the T7 RNA polymerase driven expression system (section 2.10). Thirteen mutated proteins were expressed successfully in this system. Fig.3.12 shows the result of the thirteen expressed protein analysed in a 15% SDS-PAGE. Expressed proteins are shown as swollen protein bands and the molecular weight of the protein ranged from 6,000 to 27,000. The mutated proteins observed were actually fusion proteins which contained 2 to 36 transposon-encoded amino acids at its C-terminus. The numbers of amino acids added are shown in the following table:

Tn*1000* end	frameshift[a]	additional amino acids
delta end	0	2
	1	36
	2	12
gamma end	0	2
	1	17
	2	36

[a]number of bases between last complete codon of target sequence and start of Tn*1000*.

The mutated proteins were then analysed by Western blotting.

3.2.8 Analysis of TCS deletion mutants by Western blotting

The mutated proteins were analysed by Western blotting for the presence of antigenic epitopes. The total cell proteins were first applied to a 15% SDS-PAGE (sections 2.2.12 and 2.11). After electrophoresis, the proteins were transferred to a NC membrane for the binding of anti-TCS antibody (section 2.2.14)

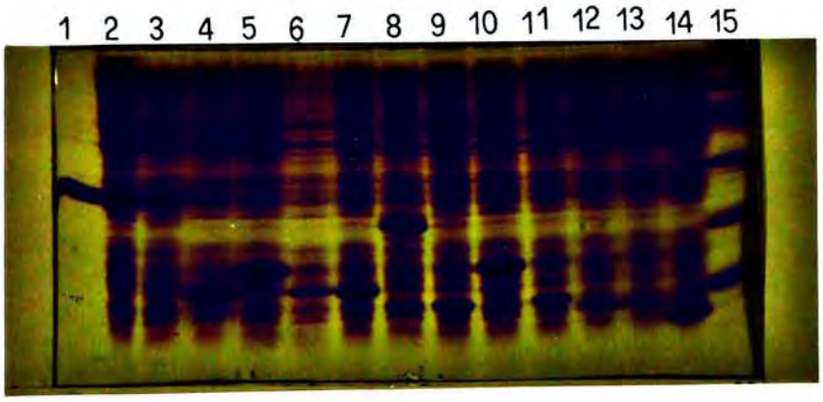


Fig. 3.12. SDS-PAGE showing the successfully expressed TCS deletion mutants. Lane (1): purified TCS, lane (2): natural TCS, lane (3): mutant 38, lane (4): mutant 308, lane (5): mutant 86, lane (6): mutant 101, lane (7): mutant 4, lane (8): mutant 201, lane (9): mutant 163, lane (10): mutant 243, lane (11): mutant 277, lane (12): mutant 29, lane (13): mutant 21, lane (14): mutant 35 and lane (15): the molecular weight marker.

Anti-TCS polyclonal antibody analysis

Fig.3.13 shows the result of Western blotting using anti-TCS polyclonal antibody. All the thirteen mutants can react with the polyclonal antibodies as shown. The smallest deletion protein is clone 35 which contains only 21 amino acids of the N-terminal of TCS. Among the mutants, mutants 29, 21 and 35 gave a weaker signal in Western blotting than other mutated protein.

Classical anti-TCS monoclonal antibody analysis

Fig.3.14 shows the result of Western blotting if the mutated proteins interacted with a classical anti-TCS monoclonal antibody generated by hybridoma technique. All the thirteen mutants can react with the monoclonal antibody. Degradation products of the mutated proteins were not observed in the Western blot. The smallest mutated protein (no. 35) could also react with the monoclonal antibody. Mutants 29 and 21 gave the weakest signal compared to the other mutated proteins.

Recombinant anti-TCS Fab fragment

Fab fragment was prepared as shown in section 2.2. Forty two clones of the Fab fragment were produced by this method. All these Fab fragments were expressed as described in section 2.3 and interacted with the deletion mutated TCS protein. These procedures should screen out the different fragments that recognize different epitopes on the TCS protein. To our surprise, we found that all the forty four clones of Fab fragments produced the same interacting pattern in Western blotting with the truncated proteins. Fig.3.15 shows a typical result of Western blotting

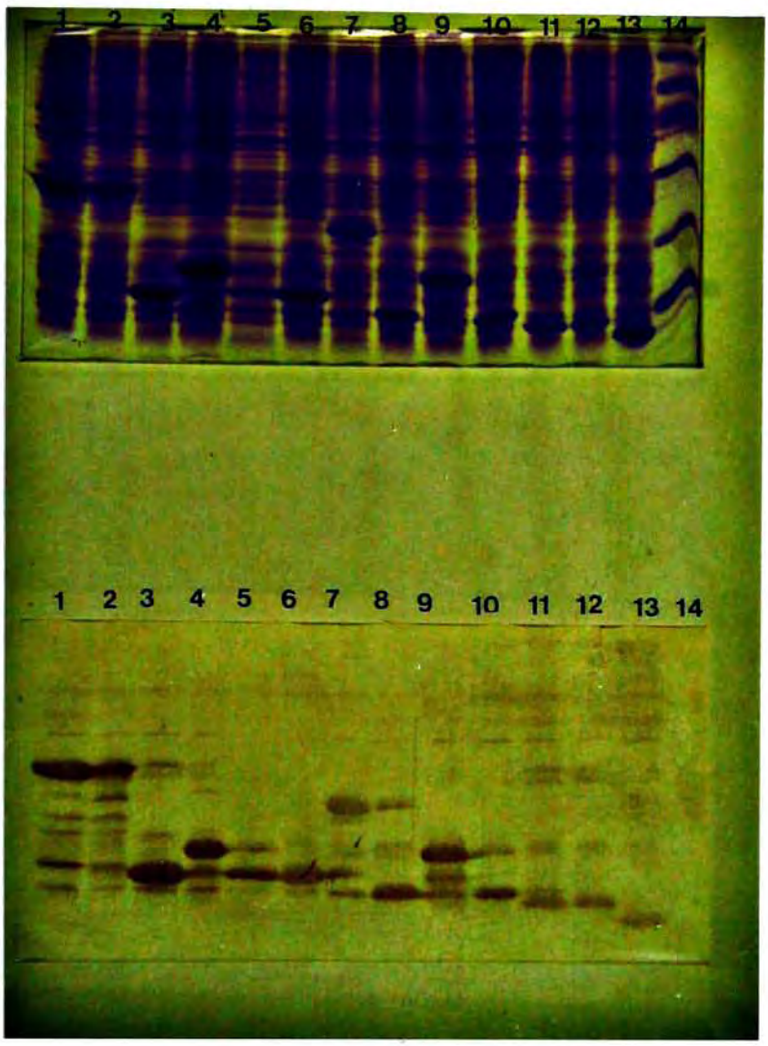


Fig. 3.13. SDS-PAGE and Western blotting analysis of TCS deletion mutants by anti-TCS polyclonal antibody. Lane (1): natural TCS, lane (2): mutant 38, lane (3): mutant 308, lane (4): mutant 86, lane (5): mutant 101, lane (6): mutant 4, lane (7): mutant 201, lane (8): mutant 163, lane (9): mutant 243, lane (10): mutant 277, lane (11): mutant 29, lane (12): mutant 21, lane (13): mutant 35 and lane (14): the molecular weight marker.

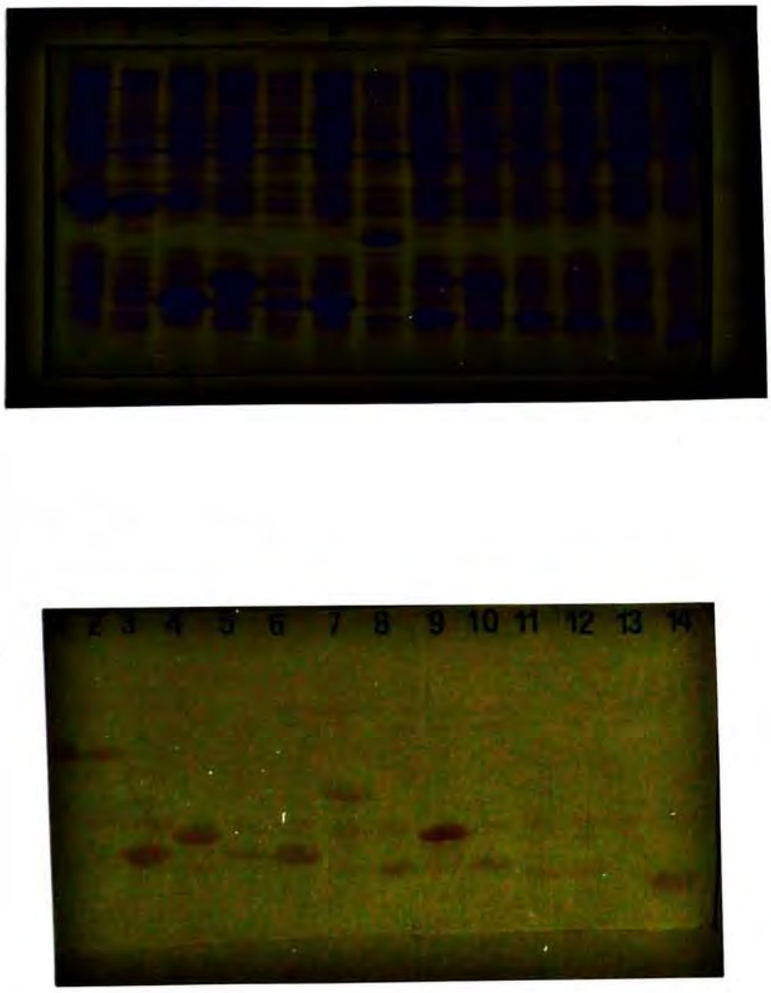


Fig. 3.14. SDS-PAGE and Western blotting analysis of TCS deletion mutants by anti-TCS classical monoclonal antibody. Lane (1): natural TCS, lane (2): mutant 38, lane (3): mutant 308, lane (4): mutant 86, lane (5): mutant 101, lane (6): mutant 4, lane (7): mutant 201, lane (8): mutant 163, lane (9): mutant 243, lane (10): mutant 277, lane (11): mutant 29, lane (12): mutant 21, lane (14): mutant 35.

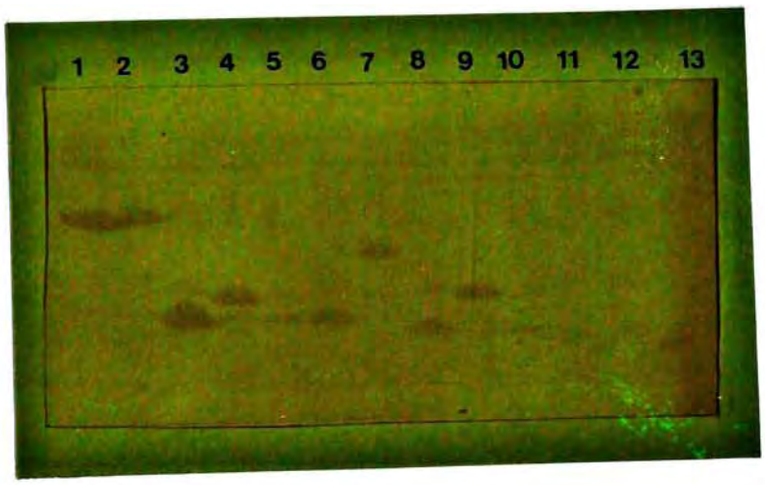


Fig. 3.15. Western blotting analysis of TCS deletion mutants by Fab fragment. Lane (1): natural TCS, lane (2): mutant 38, lane (3): mutant 308, lane (4): mutant 86, lane (5): mutant 101, lane (6): mutant 4, lane (7): mutant 201, lane (8): mutant 163, lane (9): mutant 243, lane (10): mutant 277, lane (11): mutant 29, lane (12): mutant 21 and lane (13): mutant 35.

using anti-TCS Fab fragment. The result was also similar to that of classical monoclonal antibody. As in the previous case, the smallest mutated protein (no.35) could also react with the Fab fragment and mutants 29 and 21 interacted much weaker with the Fab fragment.

3.2.9 Construction of deletion mutants that exclude the 21 amino acids in the N-terminal of TCS

In view of mutant 35 which contains the first 21 amino acid residues of TCS can still interact with polyclonal, monoclonal and Fab fragment, mutants with the deletion of the first 21 amino acid residues were constructed. The methodology was shown in section 2.9 and the strategies of the construction are shown in Fig.3.16. The results of expression and Western blotting are shown in Fig.3.17. With the exclusion of the first 21 amino acids, the mutants can still bind with the polyclonal, monoclonal and Fab fragments.

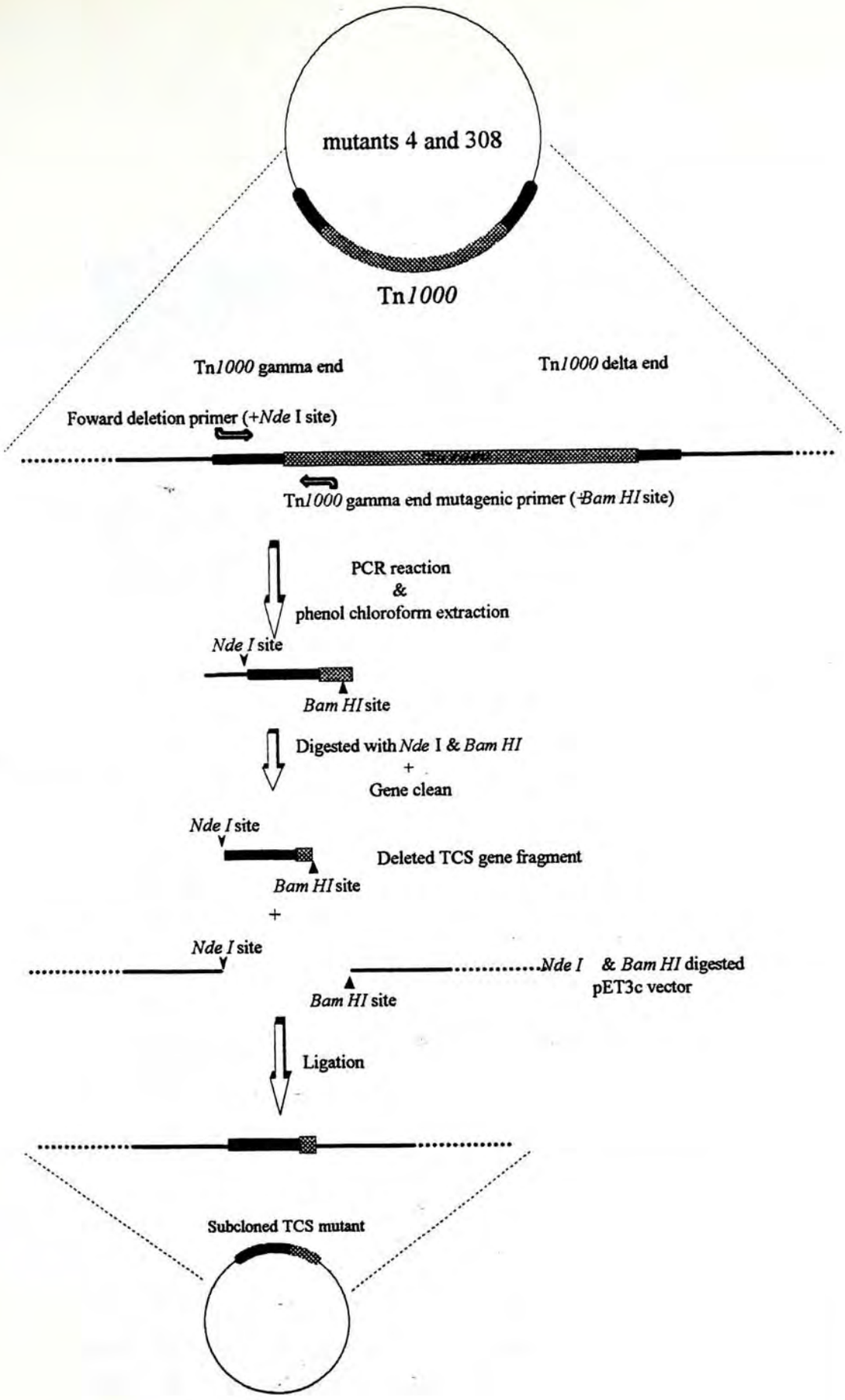
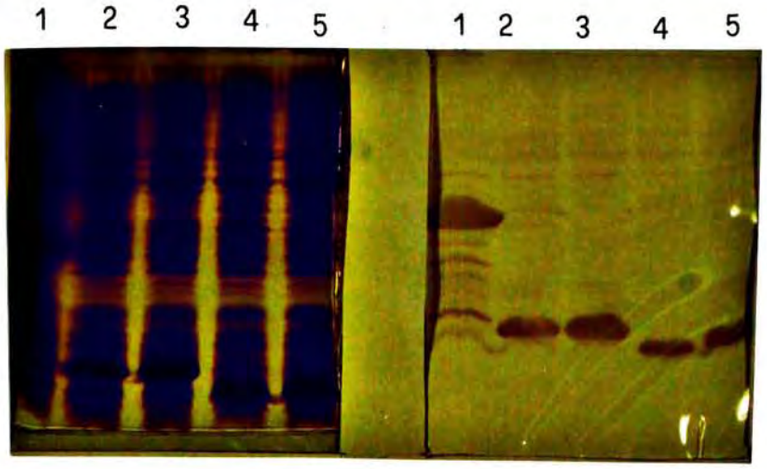


Fig.3.16 Subcloning of the deletion mutants without the 21 amino acids at the N-terminal of TCS

Anti-TCS polyclonal antibody



Anti-TCS classical monoclonal antibody

Anti-TCS Fab fragment

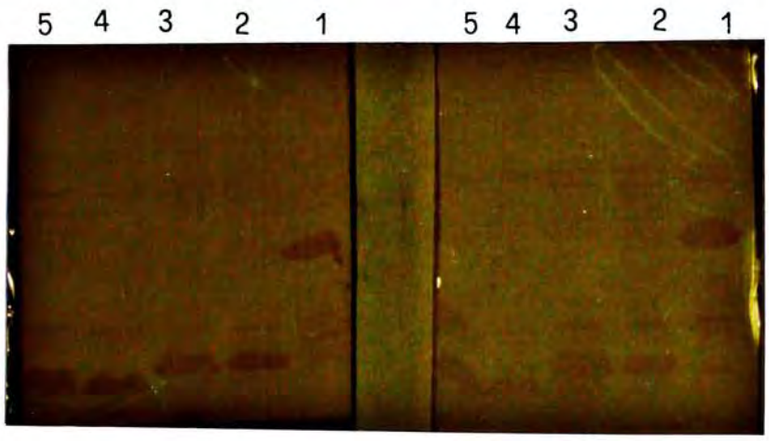


Fig. 3.17. SDS-PAGE and Western blotting of mutant 4 and 308 which have their N-terminal 21 amino acids deleted. They were analysed by polyclonal antibody, monoclonal antibody and Fab fragment. Lanes (2): and (3): are mutants 4 and 308 respectively. Lanes (4): and (5): are mutants 4 and 308 with deletion of the 21 amino acids respectively. Lane (1): is natural TCS.

3.3 Discussion

In recent years transposable elements have been increasingly exploited as tools for mutational analysis and *in vivo* genetic engineering in a diverse array of microorganisms. Among their major uses are insertion mutagenesis markers; analysis of transcriptional and translational regulation and protein localization; the introduction of engineered genes into organisms; and DNA sequencing (Berg *et al.* 1989). In this study, transposon mutagenesis was used to construct deletion mutants of TCS protein. The mutants were then reacted with antibodies to define epitopes on TCS protein. Similar work has been done by Sedgwick *et al.* (1991) to map for the epitopes of muscular dystrophy protein. Tn*1000* was used for insertion mutagenesis is because insertion mutagenesis can be performed in very simple procedures with little *in vitro* manipulation. In this study, deletion mutants of TCS gene sequence can be produced by simple transformation and bacterial mating.

One problem involved in the use of Tn*1000* as mutagenic agents is that extensive screening procedures are required for the finding of the right mutated clones (Strathmann *et al.*, 1991). As stated in the result section (3.2.2), theoretically only 10% of the mutated clones possesses a Tn*1000* inserted within the TCS gene sequence. Therefore a rapid and simple procedure was required to screen for a large number of mutated clones to identify the target mutants. In this study PCR insertion mapping was used. Other methods of screening are also available. For example the use of restriction enzyme mapping for the mutated plasmid. However problems have raised in finding the suitable restriction site and in the preparation of a large amount of plasmid DNA (Strathmann *et al.*, 1991). On the

contrary, PCR reaction requires small amount of substrate and can screen for a large amount of mutants in a short period. After PCR insertion mapping, it was shown that only 5% of the mutants contain the Tn*1000* inserted within the TCS gene sequence compared to 10% of the theoretical value. This may be due to the fact that Tn*1000* has an insertion preference for the high AT content in local sequence (Sherratt, 1989), therefore variation in insertion frequency may presence in different regions of the sequence.

Another problem encountered in this project is the expression of the protein in T7 RNA polymerase driven expression system. After the screening and sequencing of the useful mutants (Tn*1000* inserted within TCS gene sequence), the mutants were expressed in the T7 RNA polymerase driven expression system. However, the expressed product was the wild-type TCS protein as shown by Western blotting (Fig. 3.9) and protein finger-printing (result not shown). We are not sure of the exact reason. Contamination of the mutants with the pET58210 plasmid was not possible because in agarose gel electrophoresis, only single DNA bands were observed for the mutants with different molecular sizes compared to pET58210. Contamination of mutated plasmid with Tn*1000* inserted outside TCS gene sequence was also not possible because in sequencing reaction, only one exact sequence was observed and single colony was picked for expression of mutants. Therefore the remaining explanation is that Tn*1000* is not stable in the expression host (BL21 DE3 pLysS) for some reasons and hence leads to the recombination of the mutants and restores the normal pET58210 plasmid. However *in vivo* excision of Tn*1000* is rare as Tn*1000* is quite a stable transposon (Sherratt, 1989).

Also, if this is the case, owing to the duplication of the insertion site, it is highly impossible that the TCS DNA is still in frame and leads to the expression of full length protein. Nevertheless, owning to limitation of time, this phenomenon was not further investigated.

Because of the possible interference of Tn*1000* on the expression of the deletion mutants, subcloning procedures were followed to eliminate Tn*1000* in the expression system. Two subcloning procedures were employed to adopt for different insertion orientation of Tn*1000* in the TCS gene sequence. The strategies of both subcloning procedures are outlined in 3.2.6. In both approaches, the translation stop codons of the Tn1000 in delta end or gamma end were included. This aims at to provide a translation stop codon for the expression of deletion mutants. After subcloning, the deletion mutants can be expressed in a substantial amount for easy recognition. This seems to imply that Tn*1000* was unstable in the expression host and led to unsuccessful expression of deletion mutants.

After the successful expression of 13 out of 15 deletion mutants, the mutated proteins were analysed by Western blotting with the use of anti-TCS polyclonal antibody, classical monoclonal antibody and recombinant Fab fragment.

The results of Western blotting of some mutants may give information on the antigenic sites of TCS protein and they are summarized in the following table:

	Corresponding amino acid of TCS	Result of Western blotting with polyclonal antibody	Result of Western blotting with monoclonal antibody	Result of Western blotting with Fab fragment
TCS	1-247	++++	++	++
mutant 277	1-108	++++	++	++
mutant 4F[1]	21-142	++++	++	++
mutant 29	1-96	++	+	+
mutant 21	1-93	++	+	+
mutant 35	1-21	++	++	++

+: relative reactivities between the protein and antibody.

Polyclonal antibody

From the result of polyclonal antibody analysis, all the mutants can bind with polyclonal antibody. However the three shortest deletion mutants; mutant 29, 21 and 35 showed a weaker signal in the binding towards polyclonal antibody. One possible explanation is that two groups of antibodies are presence in the polyclonal antibody. One type of antibody recognized the amino acid sequence between residues 96-108, therefore after the deletion of this region (mutants 29, 21 and 35) a weaker signal was observed in Western blotting. Another explanation is that a species of antibody in the polyclonal pool is responsible for the recognition

[1] mutant 4 that has its 21 amino acids at the N-terminal deleted.

of epitopes between 1-108, if residues 96-108 were deleted, there was a reduction in epitope numbers and hence a reduced signal. The third possibility is that deletion of the residues 96-108 perturbed the conformation of epitopes recognized by the concerned antibody.

The shortest deletion mutant; mutant 35, can still bind with the polyclonal antibody. This means that this region (residues 1-21) corresponds to one of the antigenic sites recognized by polyclonal antibody. However the deletion of amino acid residues 1-21 in mutant 4 did not result in the total elimination or decrease in binding by polyclonal antibody. This means that the residues 1-21 are only a part of the antigenic site recognized by the polyclonal antibody.

Monoclonal antibody and Fab fragment

The result of Western blotting of the deletion mutants using monoclonal antibody and the forty four clones of Fab fragment were the same. This means that both the monoclonal antibody and the forty four clones of Fab fragment recognized the same antigenic site on the TCS protein. The shortest deletion mutant; mutant 35, can still bind with the monoclonal antibody and Fab fragment. This means that this region (residues 1-21) corresponding to one of the antigenic sites recognized by the monoclonal antibody and the Fab fragments. The deletion of amino acid residues 1-21 in mutant 4 did not result in the total elimination of binding by the monoclonal antibody and Fab fragment. This means that the residues 1-21 are only a part of the antigenic site recognized by the monoclonal antibody and Fab fragment.

The identical antigenic site recognized by the forty four clones of Fab fragment can be explained as follow. During the process of anti-TCS Fab fragments production (section 2.3), the mouse was boosted by TCS protein for four times before sacrificed for the spleen and there might be bias in the recognition of a particular epitope by the immune system. Also, in the process of the construction of recombinant Fab fragment, there were a series of procedures to amplify the antibody gene by PCR. This process might result in the loss of a variety of different type of Fab fragments that recognized different antigenic determinants and hence resulted in the isolation of a single clone of Fab fragments.

From the result of Western blotting a weaker signal was observed in mutants 29 and 21 which correspond to the amino acid residues 1-96 and 1-93. From Fig.3.18 it can be shown that the region of the amino acid residues of 96-107 are in a central region of a well-ordered anti-parallel β-sheet structure. Therefore destruction of this region may result in affecting the binding affinity towards the monoclonal antibody and the Fab fragments. After the total deletion of the region of the anti-parallel β-sheet structure as shown in mutant 35 (residues 1-21) in Fig.3.19, the Western blotting signal became normal again.

Based on the above discussion on the result of Western blotting by monoclonal antibody and Fab fragment, it can be concluded that in early discussion the weakening of Western blotting result in mutants 29, 21 and 35 with the use of polyclonal antibody is due to the perturbation of the conformation of epitopes recognized by the concerned antibody. Therefore from the result of transposon mediated deletion mutagenesis epitopes

mapping, it can be concluded that a discontinuous epitope is presence on TCS protein which located between residues 1-21 and 21-142.

Fig. 3.18. Computer generated 3D-structure of TCS. Residues 96-104 are shown in red.

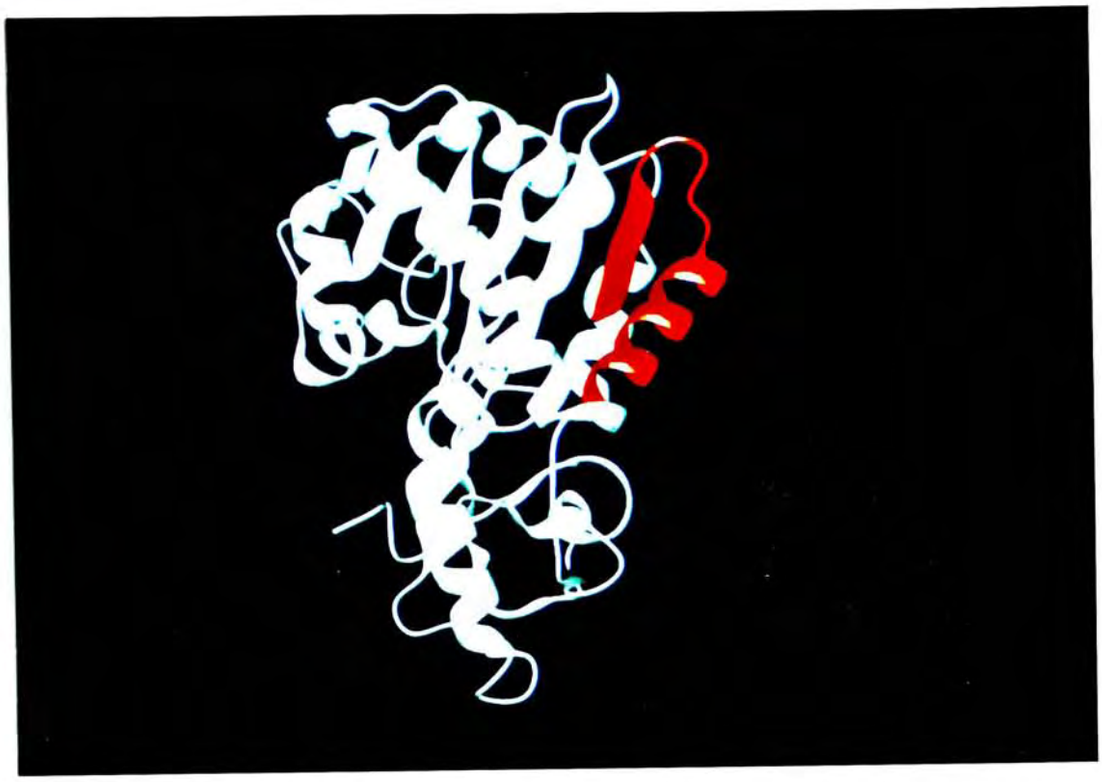


Fig. 3.19. Computer generated 3D-structure of TCS.
Residues 1-21 are shown in red.

Chapter Four

The Study of Antigenic Determinants on TCS by Chemical and Enzymatic Cleavages of TCS Protein.

4.1 Introduction

The second approach for studying the antigenic determinants of TCS protein is to produce different fragments by means of chemical or enzymatic method and then analyse by Western blotting by different antibodies.

The enzymatic method includes the use of alkaline protease, endoprotease Lys-C and endoprotease Glu-C. They are the enzymes commonly used in the cleavage of protein to produce fragments for further structure or sequence analysis. Alkaline protease cleaves at random site of the protein in alkaline condition. Endoprotease Lys-C cuts specifically at lysine peptide bonds under alkaline condition. Endoprotease Glu-C from *Staphylococcal protease* strain (V8) cleaves specifically at glutamyl peptide bonds and sometimes at aspartyl peptide bonds. These enzymes are active in alkaline condition with the presence of 1% SDS.

For chemical cleavage, cyanogen bromide was used. Cyanogen bromide has been commonly used to produce protein fragments for protein sequencing. It specifically cleaves at methionine residues in the protein sequence. Because the occurrence of methionine residue in protein is low, large protein fragments can be generated. The mechanism of cyanogen bromide cleavage at methionine is shown in Fig. 4.1. Methionine residues are converted into a mixture of C-terminal homoserine residues and homoserine lactone residues, which are inter-convertible.

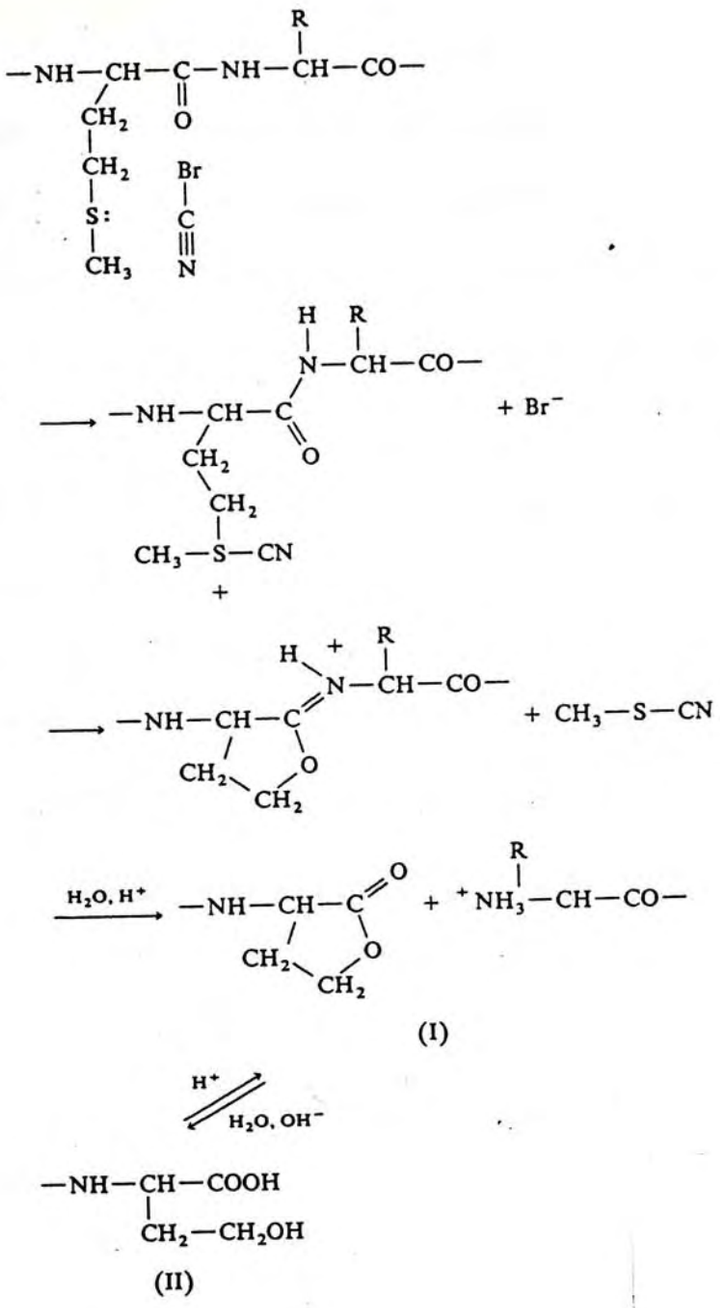


Fig.4.1. Mechanism of the reaction of cyanogen bromide on methionine

4.2 Results

4.2.1 Preparation of recombinant TCS protein

In order to provide the substrate for fragment production in the analysis of antigenic site on TCS, pure recombinant TCS protein was produced in large quantity. Recombinant TCS protein was expressed in a T7 RNA polymerase driven expression system (Zhu *et al.*,1992).

Cell lysate from 1 litre induced bacterial culture was loaded to a CM-sepharose column according to section 2.12. A gradient of 0-0.5M NaCl was applied and the elution profile of TCS is shown in Fig.4.2. Then TCS was further purified through a second CM-sepharose column with a gradient of 0-0.5M NaCl and the elution profile is shown in Fig.4.3. After lyophilization, about 25-30mg of TCS protein was collected.

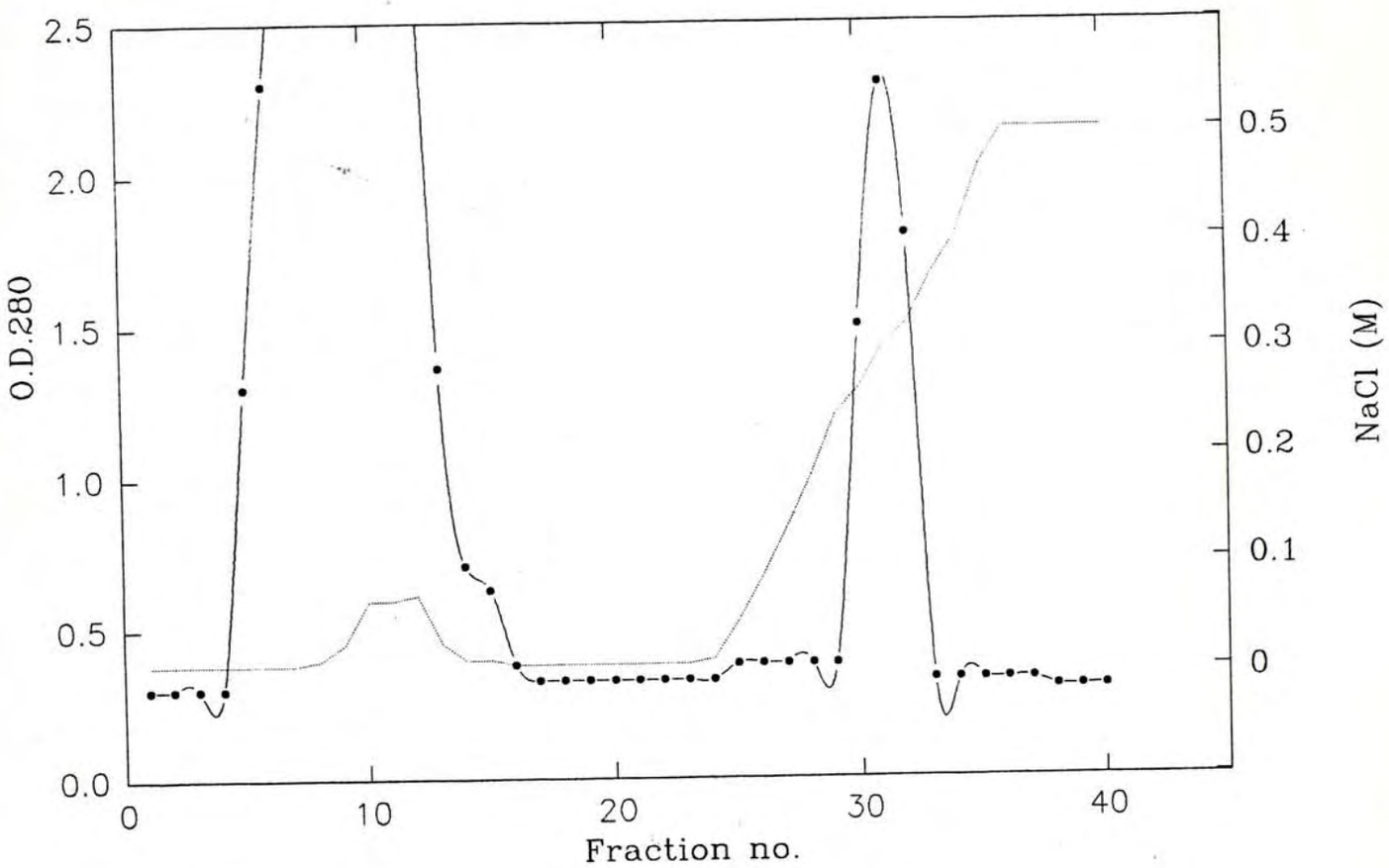


Fig.4.2. Elution profile of TCS in the first CM–sepharose column.

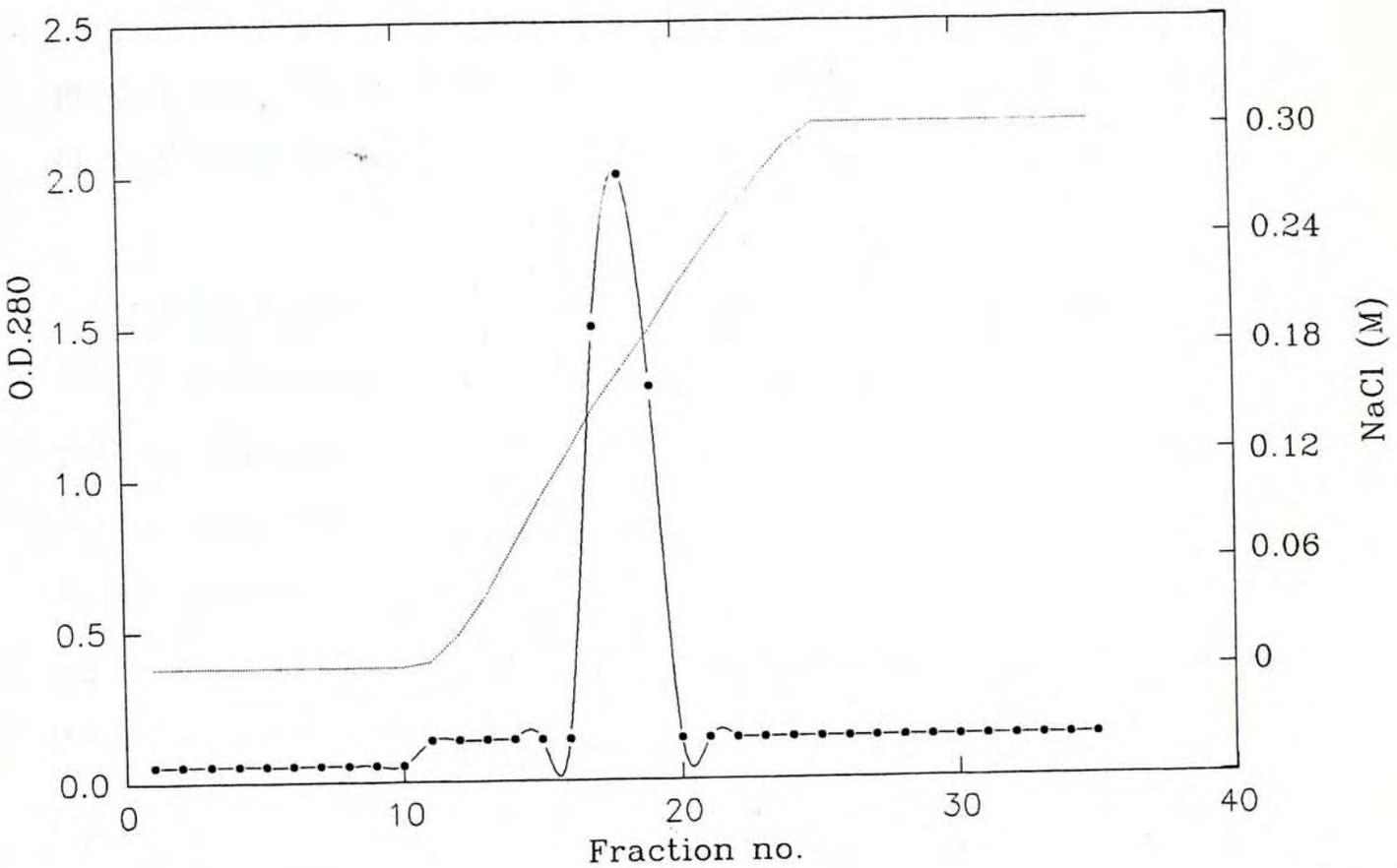


Fig.4.3. Elution profile of TCS in the second CM–sepharose column.

4.2.2 Proteolytic and chemical cleavages of recombinant TCS

After digestion of TCS protein with alkaline protease, endoprotease Lys-C and endoprotease Glu-C (section 2.13), the digestion mixtures were then analysed by 16.5% high resolution Tricine SDS-PAGE (section 2.14).

After TCS protein has reacted with cyanogen bromide, the reaction product was dried by lyophilization. The dried powder was then analysed by 16.5% high resolution Tricine SDS-PAGE.

Fig.4.4 shows the result of 16.5% high resolution Tricine SDS-PAGE analysis of the proteolytic and chemical cleavage product of TCS protein. The digestion of TCS with alkaline protease generated two distinct small peptide fragments. The digestion of TCS with endoprotease Lys-C generated a large number of protein fragments with different molecular sizes. The digestion of TCS with endoprotease Glu-C generated fewer protein fragments when compared to endoprotease Lys-C digestion. The cleavage of TCS with cyanogen bromide generated four distinct protein fragments.

Because the banding pattern of the low-range molecular markers did not correspond to their molecular weights as given by the supplier, we could not accurately determine the molecular sizes of the fragments generated by proteolytic and chemical cleavage. Nevertheless, the cyanogen bromide products were sent to Dr. R. Aplin for mass determination and sizes of the bands of C1-C4 are shown in table 4.1.

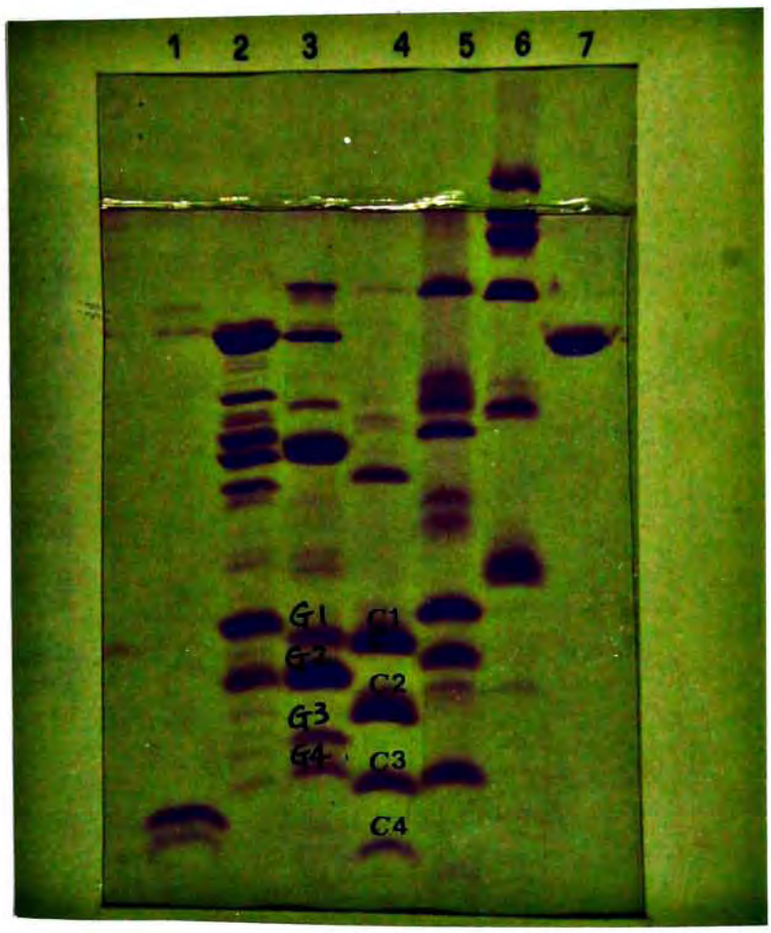


Fig. 4.4. The TCS cleaved products analysed by high resolution 16.5% Tricine SDS-PAGE. Lane (1) alkaline protease digested product, lane (2) endoprotease Lys-C digested product, lane (3) endoprotease Glu-C digested product, lane (4) cyanogen bromide cleavage product, lane (5) low-range molecular weight marker (Promega), lane (6) mid-range molecular weight marker (Pharmacia), lane (7) purifed TCS. C1, C2, C3 and C4 are the protein fragments that had been analysed by N-terminal determination and mass spectrometry. G1, G2, G3 and G4 are the interested Glu-C digested fragments.

4.2.3 Western blotting analysis of TCS protein fragment generated by proteolytic and chemical cleavage

After electrophoresis using high resolution 16.5% polyacrylamide gel, Western blotting was performed to analyse the antigenic characteristic of these fragments. Anti-TCS polyclonal antibody, monoclonal antibody and recombinant Fab fragment were used.

Polyclonal antibody

With reference to Fig.4.4, most of the fragments generated by various proteases and cyanogen bromide could bind with the polyclonal antibodies (Fig.4.5). Only the smallest fragment of the Glu-C digested and cyanogen bromide cleavage products did not react with anti-TCS polyclonal antibody.

Classical monoclonal antibody

Fig.4.6 shows the result of Western blotting with the use of anti-TCS classical monoclonal antibody. Fewer bands were observed as compared to Western blotting using polyclonal antibody. The digestion product of alkaline protease did not react with monoclonal antibody. Glu-C digested product and cyanogen bromide cleavage product revealed distinct bands while there were many partially digested fragments for Lys-C.

Recombinant Fab fragment

Fig.4.7 shows the result of Western blotting with the use of Fab fragment. Results were similar to the classical monoclonal antibody.

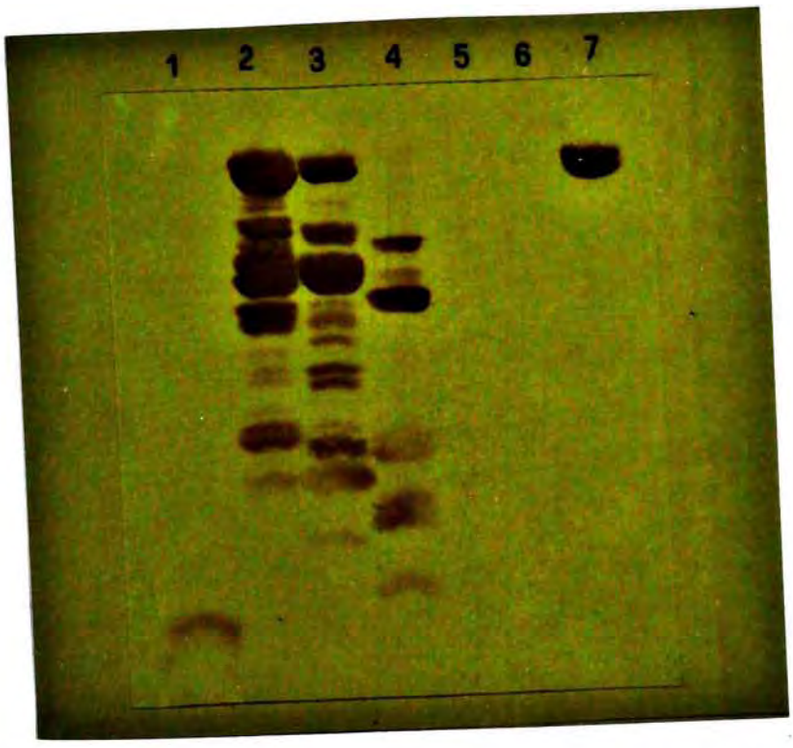


Fig. 4.5. The TCS cleaved products analysed by Western blotting with anti-TCS polyclonal antibody. Lane (1) alkaline protease digested product, lane (2) endoprotease Lys-C digested product, lane (3) endoprotease Glu-C digested product, lane (4) cyanogen bromide cleavage product, lane (5) low-range molecular weight marker (Promega), lane (6) mid-range molecular weight marker (Pharmacia), lane (7) purifed TCS.

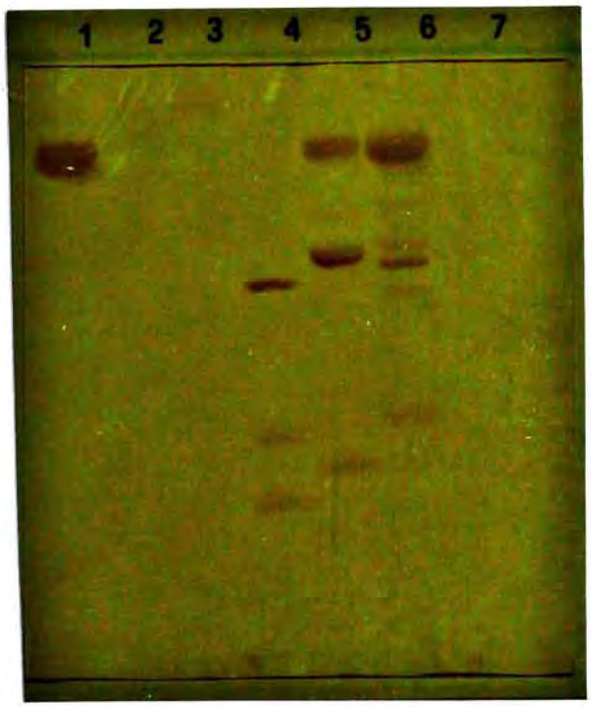


Fig. 4.6. The TCS cleaved products analysed by Western blotting with anti-TCS classical monoclonal antibody. Lane (1) purifed TCS, lane (2) mid-range molecular weight marker (Pharmacia), lane (3) low-range molecular weight marker (Promega), lane (4) cyanogen bromide cleavage product, lane (5) endoprotease Glu-C digested product, lane (6) endoprotease Lys-C digested product, lane (7) alkaline protease digested product.

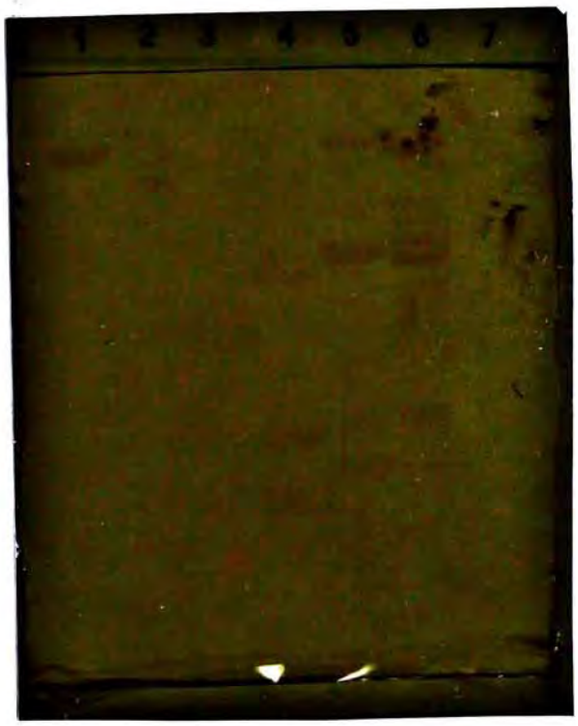


Fig. 4.7. The TCS cleaved products analysed by Western blotting with anti-TCS recombinant Fab fragment. Lane (1) purifed TCS, lane (2) mid-range molecular weight marker (Pharmacia), lane (3) low-range molecular weight marker (Promega), lane (4) cyanogen bromide cleavage product, lane (5) endoprotease Glu-C digested product, lane (6) endoprotease Lys-C digested product, lane (7) alkaline protease digested product.

Nevertheless, Fab fragment gave a much weaker signal comparing to monoclonal antibody.

4.2.4 N-terminal Determination of the antigenic TCS protein fragment produced by chemical cleavage

Because the cyanogen bromide cleavage of TCS resulted in the production of distinct protein fragments, these fragments were further analysed by N-terminal determination.

Protein fragments of TCS produced by cleavage were separated by SDS-PAGE, then blotted to a PVDF membrane (section 2.13) and sent for N-terminal determination.

Apart from N-terminal determination of the cyanogen bromide cleaved TCS protein fragments, the molecular weight of the cleavage product was determined by mass spectrometry by Dr. Robin Aplin at the University of Oxford. The results of N-terminal determination and the mass spectrometry are shown in the following table 4.1. Because of cyanogen bromide cleaves specifically at the methionine residue and there are only five methionine residues in the TCS protein, the length of the fragments can be predicted precisely.

Cyanogen bromide cleaved fragment	The N-terminal amino acids of the fragment	Predicted residues in TCS[1]	Theoretical molecular weight[2]	MS[3] determined molecular weight	React with polyclonal antibody	React with monoclonal antibody	React with Fab fragment
C1	fragment 1: Met-Asp-Val-Ser-Phe-Arg-	1-100	11261	11413	yes	yes	yes
	fragment 2: Val-Leu-Ile-Gln-Ser-Thr-	154-247	10344	10358	yes	yes	yes
C2	fragment A: Gly-Tyr-Arg-Ala-Gly-	91-153	8885	9137	yes	no	no
	fragment B: Met-Asp-Val-Ser-Phe-	1-73	8154	8195	yes	yes	yes
C3	Arg-Lys-Val-Thr-Leu-Pro-	102-153	5646	5648	yes	no	no
C4	Gly-Tyr-Arg-Ala-Gly-Asp-	74-100	3256	3308	no	no	no

Table 4.1. The N-terminal Sequences and molecular weights of the cyanogen bromide digested products of TCS: C1, C2, C3 and C4.

[1] Predicted from the N-terminal sequence, Met residue in TCS and the MS determined molecular weight.
[2] Calculated from the predicted residue of the fragment in TCS.
[3] Mass spectrometry.

Glu-C digested fragments	Proposed molecular weight [4]	React with polyclonal antibody	React with monoclonal antibody	React with Fab fragment
G1	~11,000	yes	no	no
G2	~10,500	yes	yes	yes
G3	~9,500	yes	no	no
G4	~9,000	no	no	no

Table 4.2. Proposed sizes of Glu-C fragments and their interaction with various antibodies

[4] By comparing with the molecular weight of the cyanogen bromide cleaved product

4.3 Discussion

Proteolytic and chemical cleavage of protein into smaller fragments have been commonly used in protein sequencing. In this study these approaches were used to produce TCS protein fragments for subsequent antigenic analysis.

In the study of the protein fragments, a 16.5% high resolution Tricine SDS-PAGE (section 2.12) was used. In the preliminary study, other gel systems were used for the separation of small molecular weight fragments produced by proteolytic or chemical cleavage. They included 15% and 20% SDS-PAGE (section 2.1.12), precasted 18% SDS-PAGE and 4-20% gradient gel (NOVEX). However only the 16.5% high resolution Tricine SDS-PAGE gave a satisfactory result on the separation of the protein fragments. One disadvantage of this gel system is the long preparation time in gel setting and the gel needed to run slowly to produce the required resolution and to prevent overheating.

Fig.4.4 shows that both proteolytic and chemical approaches can produce different TCS fragments. The digestion products of TCS by alkaline protease and endoprotease Lys-C were not suitable for further analysis. This is because the digestion of TCS by alkaline protease was too complete and no distinct fragment was observed. On the contrary the digestion of TCS by endoprotease Lys-C gave too many partially digested fragments. On the other hand both the Glu-C and the cyanogen bromide digestion gave distinct protein fragments for further analysis. Proposed sizes of Glu-C fragments G1, G2, G3, and G4 and their interactions with various antibodies are listed in Table 4.2. Owning to the limitation of time, the location of these Glu-C products had not been mapped for this thesis.

Fragments generated by cyanogen bromide cleavage could be resolved in 16.5% TricineSDS-PAGE (Fig.4.4). Therefore further study was done on these fragments to investigate their molecular sizes and N-terminal sequences. The cyanogen bromide cleaved fragments were marked as C1, C2, C3 and C4 (Fig.4.4). These fragments were then sent for N-terminal determination and mass analysis. The fragments were then mapped on the TCS amino acid sequence (Fig.4.8)

C1 actually contains two fragments: 1 and 2. Fragment 1 corresponds to amino acids 1-100 of TCS. It is actually the incomplete digestion product which contain C2 fragment B and C4. Fragment 2 of C1 corresponds to amino acids 154-247 of the TCS protein. All these two fragments of C1 could bind with all three types of antibody (Fig.4.5, 4.6 & 4.7).

C2 actually contains two fragments: A and B. They could be separated for N-terminal determination because two distinct bands were observed in C2 after the PVDF membrane was stained. Fragment A of C2 corresponds to residues 91-153 of TCS protein and it is actually the incomplete digestion product which contain C3 and C4. By comparing the result of Western blotting of C2 fragment A with polyclonal antibody (Fig.4.5), monoclonal antibody (Fig.4.6) and Fab fragment (Fig.4.7), it was shown that this fragment could only recognize by polyclonal antibody. Fragment B of C2 corresponds to the residues 1-72 of TCS protein. This fragment could bind with all three types of antibody.

*** SEQUENCE LIST *** (N =====> C)

10
N- Met Asp Val Ser Phe Arg Leu Ser Gly Ala Thr Ser Ser Ser Tyr Gly Val Phe
20 30
Ile Ser Asn Leu Arg Lys Ala Leu Pro Asn Glu Arg Lys Leu Tyr Asp Ile Pro
40 50
Leu Leu Arg Ser Ser Leu Pro Gly Ser Gln Arg Tyr Ala Leu Ile His Leu Thr
60 70
Asn Tyr Ala Asp Glu Thr Ile Ser Val Ala Ile Asp Val Thr Asn Val Tyr Ile
80 90
Met Gly Tyr Arg Ala Gly Asp Thr Thr Tyr Phe Phe Asn Glu Ala Ser Ala Thr
100
Glu Ala Ala Lys Tyr Val Phe Lys Asp Ala *Met Arg Lys Val Thr Leu Pro Tyr*
110 *120*
Ser Gly Asn Tyr Glu Arg Leu Gln Thr Ala Ala Gly Lys Ile Arg Glu Asn Ile
130 *140*
Pro Leu Gly Leu Pro Ala Leu Asp Ser Ala Ile Thr Thr Leu Phe Tyr Tyr Asn
150 **160**
Ala Asn Ser Ala Ala Ser Ala Leu **Met Val Leu Ile Gln Ser Thr Ser Glu Ala**
170 **180**
Ala Arg Tyr Lys Phe Ile Glu Gln Gln Ile Gly Lys Arg Val Asp Lys Thr Phe
190
Leu Pro Ser Leu Ala Ile Ile Ser Leu Glu Asn Ser Trp Ser Ala Leu Ser Lys
200 **210**
Gln Ile Gln Ile Ala Ser Thr Asn Asn Gly Gln Phe Glu Ser Pro Val Val Leu
220 **230**
Ile Asn Ala Gln Asn Gln Arg Val Thr Ile Thr Asn Val Asp Ala Gly Val Val
240
Thr Ser Asn Ile Ala Leu Leu Leu Asn Arg Asn Asn Met Ala *** -C

Fig. 4.8. TCS amino acid sequence showing the cyanogen bromide cleaved fragments. Normal text, C2 fragment B. Underlined text, C4. *Italic text*, C3. **Bold text**, C1 fragment 2.

C3 corresponds to residues 102-153 of TCS protein and it only interacted with the polyclonal antibody.

C4 corresponds to amino acids 74-100 of TCS protein and it did not interact with all the three types of antibody.

From the results of Western blotting of these digestion products with monoclonal antibody and Fab fragment, it can be concluded that these antibodies are similar and recognize a discontinuous epitope. This epitope involves the sequence of the anterior part and posterior part of the TCS protein. The anterior part includes amino acid residues of 1-73 and the posterior part include residue of 154-247. Whereas the central region of TCS protein residue 74-153 (C3-C4) is not involved.

On the other hand, an epitope was located by the polyclonal antibodies between residues 102-153 (Table 4.1). Also the interaction of C2 fragment A with polyclonal antibodies may be due to its epitope at 102-153.

Residues 74-100 seems to have no epitopes as all the three kinds of antibodies did not interact with it.

Chapter Five

General Discussion and Future Study

5.1 General Discussion

In the study of the antigenic determinants of TCS protein, two approaches have been used, the first one is the construction of deletion mutants by transposon mediated mutagenesis developed by Sedgwick *et al.* (1991). They successfully mapped the epitopes for the central helical region of muscular dystrophy. Another similar work using exonuclease III deletion instead of transposon mediated deletion mutagenesis was done by Gross and Rohemann (1990). They constructed the deletion mutants of baculovirus capsid protein and then mapped for the epitopes within the protein. Deletion can be done by exonuclease III at both C-terminal and N-terminal of the protein. A limitation of using transposon mutagenesis is that when an epitope covers different regions of a protein, such as in our case, all the deletion mutants will interact with the antibody. Hence only the antigenic site at the N-terminal of the protein can be unambiguously located.

As shown in our study, because the epitope recognized by the monoclonal antibody is discontinuous (section 4.3). Only the first 21 amino acids at the N-terminal region of TCS can be located as antigenic site. Hence we have to switch to another approach.

The analysis of the antigenic properties of proteolytic and chemical cleavage fragment of protein has been used for some time for the analysis of epitopes. This approach is especially useful when the DNA sequence of the interested protein is not available. For example Morris (1989), had

successfully identified an antigenic region of 53 amino acids at the C-terminal of chicken creatine kinase with the use of cyanogen bromide and proteinase K. Man *et al.* (1991) used nitrothiocyanobenzoic acid and proteinase K also identified a 53 amino-acid antigenic fragment in human brain creatine kinase. One characteristic of this approach is that usually the identified antigenic fragment is very large and further isolation and digestion of the concerned fragment is needed.

Chemical and enzymatic analysis of TCS protein have also been reported previously. Wang (1985) used cyanogen bromide to produce four TCS protein fragments for subsequent analysis but no epitope mapping data were reported. Ke *et al.* (1988) used chymotrypsin to produce protein fragments from TCS for epitope mapping and they found that amino acid residues of 101-247 contain an epitope.

The location of the epitopes in TCS has been refined in our study as compared to Ke et al. (1988). By comparing the results of peptide fragmentation, deletion mutagenesis epitope mapping and the epitope prediction according to Jameson-Wolf, a clearer picture of the antigenic sites of TCS protein is produced.

The following table summarizes the information on the antigenic determinants of TCS protein found by the two mentioned approaches.

Antigenic site recognized by :	Antigenic site identified by deletion mutagenesis epitope mapping (amino acids residue of TCS)	Antigenic site identified by fragmentation epitope mapping (amino acids residue of TCS)
Monoclonal antibody and Fab fragment	1-21, 21-142	1-73, 154-247
Polyclonal antibody[a]	-	102-153

[a] antigenic site recognized by polyclonal antibody only.

We have also predicted the antigenic sites of TCS using a computer program in DNASIS according to the method of Jameson-Wolf. The following table is a summary of the experimental and predicted results.

Antigenic region (amino acids residues)	recognized by:	sequence with high antigenic index (antigenic index ≥ 1.0)	secondary structure of the underlined residues determined by X-ray crystallography
1-21	pAb, mAb and Fab	6-13	random + turn structure
21-73	pAb, mAb and Fab	24-33, 41-46	random + turn structure
154-247	pAb, mAb and Fab	172-177, 191-193, 206-208	random + turn structure
102-153	pAb	109-116	random + helix structure

pAb: polyclonal antibody
mAb: monoclonal antibody
Fab: Fab fragment

The three-dimensional structure of TCS (Fig.5.1, 5.2, 5.3 and 5.4) show the antigenic region located by deletion mutagenesis and protein fragmentation. Many residues are located on random structures which are more accessible to antibody interaction. Together with the antigenic index prediction, it is very likely that the residues underlined in the above table contained genuine epitopes.

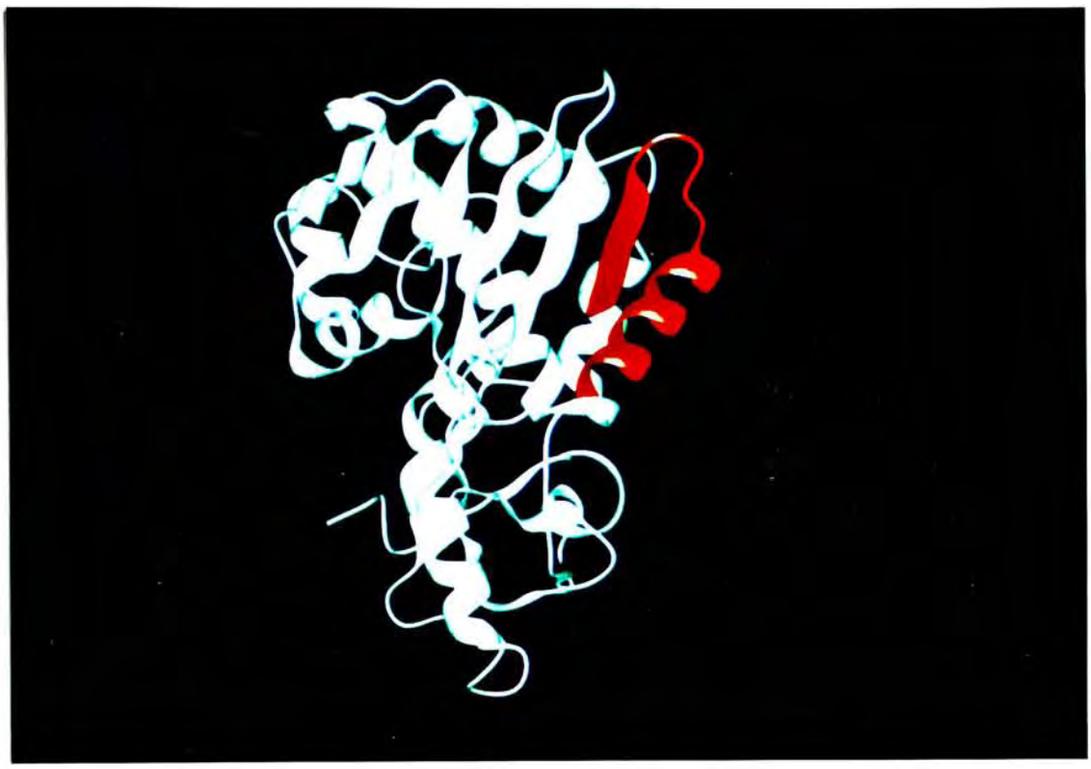


Fig. 5.1. Computer generated 3-D structure of TCS showing the antigenic residues 1-21 (red).

Fig. 5.2. Computer generated 3-D structure of TCS showing the antigenic residues 21-72 (red).

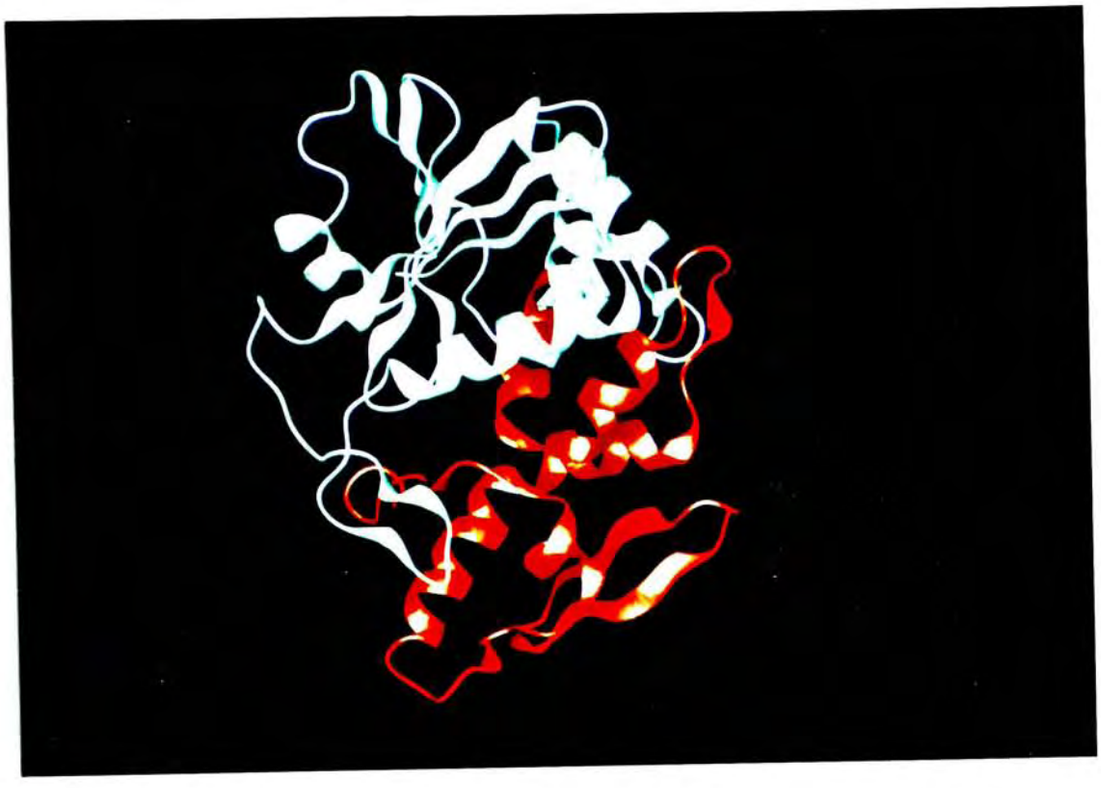


Fig. 5.3. Computer generated 3-D structure of TCS showing the antigenic residues 154-246 (red).

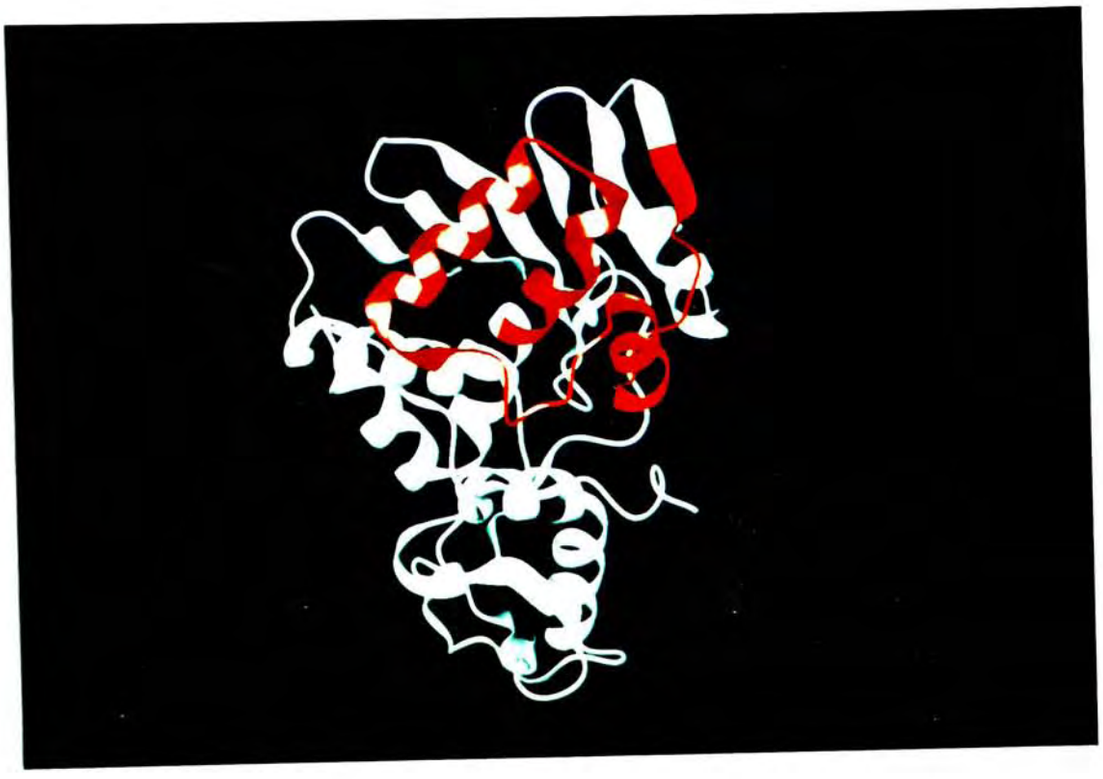


Fig. 5.4. Computer generated 3-D structure of TCS showing the antigenic residues 102-152 (red).

5.2 Future Study

Because the anti-TCS monoclonal antibody and Fab fragment recognize the anterior part and posterior part of the TCS protein, further study should be done to find out the exact location of the antigenic site in these regions. Moreover the mapping of the Glu-C digested TCS protein fragments should give more information on the antigenic site in these regions. We may also use the recombinant method to produce protein fragments with amino acid residues between 21-73, 154-247 and 102-153 for mapping of the antigenic sites involved in this region. Peptide fragments such as C1 fragment 1, C2 fragment B and C3 of TCS generated by cyanogen bromide cleavage can also be isolated by HPLC to perform further enzymatic digestion for the further characterization of the antigenic sites. Epitope library of TCS (section 1.3) can also be generated for locating discontinuous epitope on TCS protein. Moreover, different approaches to immunize the animals for generating different clones of monoclonal antibody should be used in order to find out all antigenic sites of TCS recognized by the immune system. After the immunogenic amino acid residues have been found, protein engineering technique can be used to alter the immunogenicity of TCS protein to improve its clinical performance.

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